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**ADVANCES IN ENZYMOLOGY**  
**AND RELATED SUBJECTS OF**  
**BIOCHEMISTRY**  
**Volume III**

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# ADVANCES IN ENZYMOLOGY

## AND RELATED SUBJECTS OF BIOCHEMISTRY

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VOLUME III

INTERSCIENCE  PUBLISHERS, Inc.  
NEW YORK 1943

Copyright, 1943, by  
INTERSCIENCE PUBLISHERS, INC.  
215 Fourth Avenue, New York 3, N. Y.

First Printing ..... 1943  
Second Printing (by photo-offset)..... 1945  
Third Printing (by photo-offset)..... 1947

Printed in the United States of America

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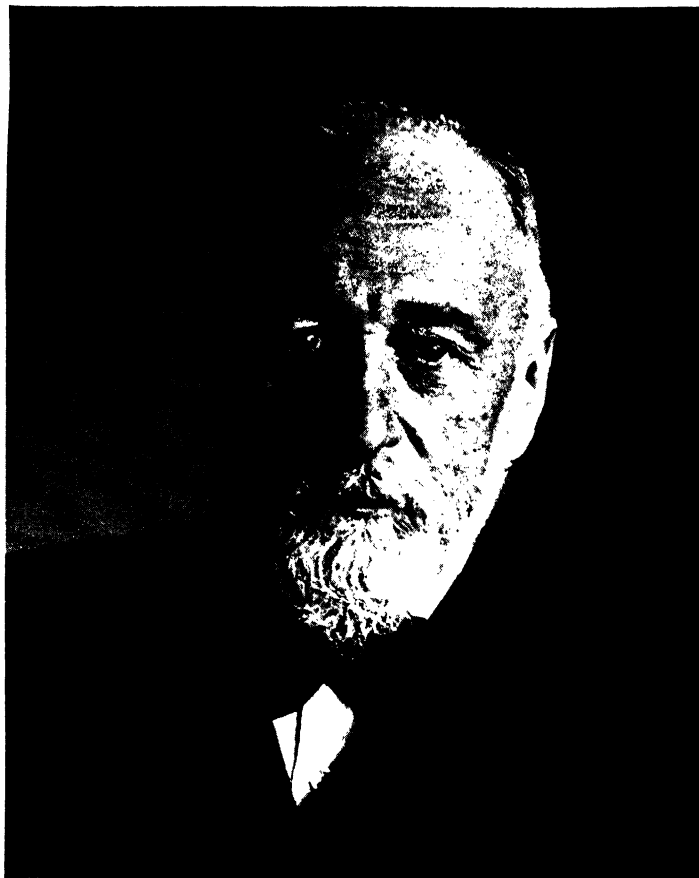
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*Ruben Willmott.*

## RICHARD WILLSTÄTTER

1872-1942

*"His life work reflects the manifold properties of matter and of the processes of living nature. With the devotion and ability of a great man of research and with veneration before the marvels of creation, he has attacked problems which were most closely connected with life, thus serving truth and humanity. The refined coinage of his personality shines out in the contributions to the scientific literature through which his accomplishments are set forth."*

ARTHUR STOLL



# CHROMOSOMES AND NUCLEOPROTEINS

By

A. E. MIRSKY

*New York, N. Y.*

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## I. Introduction

Nucleoproteins have been recognized by biochemists as components of biological systems for nearly 75 years. Their functions in the cell are only now beginning to be understood. Nucleoproteins, it is now known, occur in both nucleus and cytoplasm. In some way the nucleoproteins of the nucleus take part in the processes of reproduction that are known to occur in chromosomes. The discovery that plant viruses are self-duplicating nucleoproteins has emphasized the fact that chromosomes consist largely of nucleoprotein, and has brought forward the possibility that the nucleoproteins of chromosomes are self-duplicating.

The conception of a self-reproducing substance is more novel for the biochemist than it is for the cytologist and the geneticist. In a recent review of the role of genes in cellular physiology Sewall Wright observes that

"In textbooks of physiology, it is customary to treat the cell as the ultimate living unit. The properties of the cell are interpreted as resulting from the interactions of individually non-living substances when organized in the physical system characteristic

of protoplasm. A different treatment is found in genetics. The ultimate unit of life here is not the cell but the gene" (91).

Even before the rise of genetics it had become clearly recognized by cytologists that there are self-duplicating units within the cell itself. By the middle of the last century it had become definitely established that every cell arises by the division of a pre-existing cell; *omnis cellula e cellula* (Virchow, 1855). In the next half-century a brilliant group of cytologists laid the foundations of our knowledge of chromosomes. An account of their work is given in E. B. Wilson's great book, from which the following passage is taken (p. 828).

"In any general account of the history and genetic relations of the chromosomes in the life-cycle, we inevitably find ourselves speaking of them as if their identity were not really lost when they disappear from view in the resting or vegetative nucleus. The vast literature of the subject is everywhere colored by the implication that chromosomes, or something which they bear, have a persistent individuality that is carried over unchanged from generation to generation. This view has met with some determined opposition; but with the advance of exact studies on the chromosomes, skepticism has gradually yielded to the conviction that the chromosomes must, to say the least, be treated as if they were persistent individuals that do not wholly lose their identity at any period in the life of the cell but grow, divide and hand on their specific type of organization to their descendants. This does not mean that chromosomes are to be thought of as fixed and unchangeable bodies. Beyond a doubt they undergo complex processes of growth, structural transformation and reduction, in some cases so great that not more than a small fraction of the substance of the mother-chromosomes at its development is passed on to the daughter-chromosomes. Whether we can rightly speak of a persistent 'individuality' of the chromosomes is a question of terminology. What the facts do not permit us to doubt is that chromosomes conform to the principle of genetic continuity; that every chromosome which issues from a nucleus has some kind of direct genetic connection with a corresponding chromosome that has previously entered that nucleus."

Cytologists did not stop at even this point. They observed that chromosome threads often have a beaded appearance. These minute beads or granules of chromatin were later called *chromomeres*, and of them Wilson says (p. 906):

"We are thus brought, finally, to one of the most fundamental conceptions of cytology and genetics, namely, that the spireme-threads are linear aggregates of much smaller self-perpetuating bodies, aligned in single series, and in definite order . . . . The chromomeres, whatever their ultimate significance, are capable not only of growth, definite alignment and division, but also of conjugating two by two and like with like. To some minds, perhaps to many, this result may seem too staggering for serious consideration. If so, we may, with advantage, reflect on the fact that precisely the same

result concerning the relations of the Mendelian unit-factors of genes of heredity had been independently reached by the exact experimental methods of modern genetic analysis."

Developments in cytogenetics during the 17 years since this was written have amply confirmed this point of view; chromosomes and genes are self-propagating bodies that do not arise *de novo*.

At the time that cytologists were showing that chromosomes and even chromomeres are self-reproducing bodies, it was known that a considerable part of these structures consists of nucleoprotein. It should not be supposed that there was a clear understanding of the chemical composition of chromosomes, but it was generally agreed that the affinity of chromatin for basic dyes was due to its nucleic acid content. Cytologists were aware of Miescher's great discoveries. Miescher himself, as can be seen from his papers and letters, was interested in biological as well as chemical problems.

After the discovery of nucleic acid by Miescher, much of the investigation in this field was carried on by Kossel and Levene and their collaborators. For them, nucleic acids were complex organic substances and investigation of them became an important chapter in organic chemistry. The contributions of Kossel and Levene are, of course, invaluable for an understanding of nucleic acids, and accordingly, in the long run, for chromosomes. And, yet, it is possible that the absorption of these investigators and others whom they influenced in the problems of organic chemistry meant that for some time there was little collaboration between cytologists and biochemists in the study of chromosomes.

This period of isolation ended a number of years ago. Biochemists began to pay especial attention to those properties of nucleic acids and nucleoproteins, which would help in the study of the nucleus. In most cases this meant investigating preparations of these substances that had not been degraded by the methods of isolation, as had been the case in the preparations investigated by Miescher, Kossel and Levene. In the midst of this "back to nature" movement of the biochemists there came a momentous discovery from an unexpected quarter--the discovery that plant viruses are nucleoproteins. These bodies like chromomeres and genes are small compared with the cells in which they are reproduced; like chromomeres they are made of nucleoprotein; and like genes they mutate. The self-duplication of all these bodies appears to require the environment within the cell. Just how fruitful for research the analogy between virus and gene is, remains to be seen, but in the meantime it is hardly surprising that the analogy should be constantly in the minds of investigators.

It is perhaps surprising to find that this analogy was recognized long be-



fore the nucleoprotein nature of any virus had been discovered. The following passages are taken from a paper written in 1921:

"Finally, there is a phenomenon . . . which must not be neglected by geneticists. This is the d'Hérelle phenomenon . . . the substance (*i. e.*, the phage, or, as it is now frequently called, the bacterial virus), when applied to colonies of bacteria, become multiplied or increased, and could be so increased indefinitely; it was self-propagable. It fulfills, then, the definition of an autocatalytic substance, and although it may really be of a very different composition and work by a totally different mechanism from the genes and the chromosomes, it also fulfills our definition of a gene. . . .

"That two distinct kinds of substances—the d'Hérelle substance and the genes—should both possess this most remarkable property of heritable variation or 'mutability,' each working by a totally different mechanism, is quite conceivable, considering the complexity of protoplasm, yet it would seem a curious coincidence indeed. It would open up the possibility of two totally different kinds of life, working by different mechanisms. On the other hand, if these d'Hérelle bodies were really genes, fundamentally like our chromosome genes, they would give us an utterly new angle from which to attack the gene problem. They are filterable, to some extent isolable, can be handled in test tubes, and their properties, as shown by their effects on the bacteria, can then be studied after treatment. It would be very rash to call these bodies genes, and yet at present we must confess that there is no distinction known between the genes and them. Hence we cannot categorically deny that perhaps we may be able to grind genes in a mortar and cook them in a beaker after all. Must we geneticists become bacteriologists, physiological chemists and physicists, simultaneously with being zoologists and botanists? Let us hope so" (68).

With the isolation of plant viruses and the discovery that they are nucleoproteins, it seems as if Muller's remarkable prophecy may indeed come to pass. And yet an interest in viruses is only one of the factors that is attracting bacteriologists, biochemists and physicists to the study of chromosomes and genes. There are biochemists, as well as cytologists and geneticists, who now realize that an understanding of the minute self-duplicating particles in chromosomes is essential for the development of their science. In this paper an account is given of some of the beginnings that have been made in biochemical investigations of chromosomes and of nucleoproteins generally. There is no attempt at completeness in the account given.

## II. Feulgen's Nucleal Reaction

The first contribution of biochemistry since the work of Miescher and Kossel to have a direct and immediate value for chromosome chemistry was made by Feulgen in 1924 (34). From that time to the present the nucleal reaction for deoxyribose nucleic acid has been a useful tool. An indication of activity in this field is the size of the bibliography of the nucleal reaction,

which up to 1938 fills eighteen pages (65). The value of the nucleal reaction is its specificity. When a structure appears colored by this procedure the cytologist can not only see the structure, but he knows something about its chemical constitution—a rare event in cytology. Indeed the nucleal reaction is much more like a spot test of the chemist done inside the cell than it is like the familiar staining technique of the cytologist. In a spot test the color appears usually as a result of the reaction that occurs; in staining, a dye is used and whether or not a given structure takes the dye depends to a considerable extent on the physical state of that structure as well as on its chemical constitution.

The fuschin-sulfurous acid reagent used by Feulgen has been familiar to organic chemists since 1866 as a highly specific test for aldehydes (Schiff's test). Feulgen observed that thymus nucleic acid gives this test after rather gentle hydrolysis (and only after hydrolysis) in 1 *N* HCl, whereas yeast nucleic acid does not. He showed that the sugar present in the hydrolysate is responsible for the aldehyde test. Since substances with true aldehydic groups are very uncommon in biological material the nucleal reaction (Schiff's test after hydrolysis in 1 *N* HCl) is quite specific for thymus nucleic acid. Feulgen, himself, discovered the test ten years before he used it to stain the nucleus, and it was not until six years after its use in cytology that Levene and his collaborators finally succeeded in showing what kind of a sugar (a pentose, desoxyribose) is responsible for the test (55).

It may appear surprising that the nucleal reaction is effective cytologically because the products of hydrolysis might have been expected to diffuse away as a result of the preliminary hydrolysis in HCl. This possibility was carefully considered by Feulgen himself; he showed that there is, in fact, no such tendency for the products of hydrolysis to wander about in the cell (34).

The great importance of the nucleal reaction is that it has been the means of demonstrating that the nuclei of all cells contain desoxyribose nucleic acid. Until 1924 it was thought that although cells of animals contain this nucleic acid in their nuclei, nuclei of plant cells contain ribose nucleic acid instead. This supposed distinction between animal and plant cells vanished when Feulgen showed that the nuclei of wheat embryo cells give an intense nucleal reaction. Thirteen years later the presence of desoxyribose nucleic acid in nuclei of plant cells was made certain when this nucleic acid was isolated by Feulgen and his colleagues from the nuclei of rye embryo cells (by a method to be described later) (33).

Since Feulgen's original contribution, cytologists have greatly improved the technique of fixation and have modified other important details in the use of the nucleal reaction (4, 44). The result of these improvements has been that, whereas, at first, there was disagreement among different investigators about whether certain nuclei give a nucleal reaction, it is now generally agreed that practically all nuclei, whether of plant or animal cells, give the reaction, although occasionally, as in the nuclei of certain eggs, some react feebly (5, 11). Feulgen, himself, failed to obtain the reaction in

yeast, but here again improvements in the technique of fixation yielded positive results and indeed provided the final evidence that there is a nuclear-like body in yeast (90, 2). That nuclei of all plants and animal cells give a nucleal reaction is all the more striking when it is considered that even after careful investigation, no nucleal reaction has yet been detected in the cytoplasm of any cell.\*

The nucleal reaction has shown an essential and unsuspected chemical similarity of animal and plant chromosomes. In the case of bacteria it has shown that there exists something resembling the nuclei of cells in higher organisms. Until fairly recently there has been considerable doubt as to whether anything analogous to chromatin exists in bacteria, and many of those investigators who believed that a chromatin-like material is present supposed that it was diffused throughout the bacterial cell. Application of standard cytological technique along with the nucleal reagent has clearly shown within the past few years, that many bacteria contain nuclear-like bodies when stained by the traditional cytological nuclear dyes and that these bodies can indeed be considered analogous to true nuclear structures in that they give a positive Feulgen reaction (86, 73). A recent investigator states that a diffusely distributed chromatinic substance never occurs in bacteria and that when it is observed, this is due to faulty cytological technique (69). In most cases bacterial nuclei occur embedded within the bacterial cell but in resting spores they have been seen "closely attached to, but distinct from, a rod of non-chromatinic protoplasm" (74).

The nucleal reaction of Feulgen has provided evidence that the nuclei of all cells contain desoxyribose nucleic acid, that this nucleic acid is absent from the cytoplasm (at least in quantities detectable by this procedure), and that the nuclear-like bodies of bacteria are comparable with nuclei of other cells in that they, too, contain desoxyribose nucleic acid.

### III. Investigations of the Nucleus with the Quartz Microscope and Ultraviolet Light

T. Caspersson has carried out a magnificent series of cytochemical investigations using a quartz microscope with ultraviolet light. The method used by Caspersson depends upon the unique absorption spectrum of nucleic acid in the ultraviolet.

Figure 1 gives the absorption spectrum of nucleic acid and also that of several proteins. The position of maximum absorption and the extraordinarily high extinction

---

\* In this connection it is important to distinguish between the Feulgen nucleal and plasmal reactions.

coefficient characterize nucleic acid, distinguishing it from other substances present in considerable quantity in biological material, so that nucleic acid can be detected by its absorption in the ultraviolet even when present in a mixture of proteins and other substances, as it is in a cell.

The unusual ultraviolet absorption spectrum of nucleic acid was observed as far back as 1906 by Dhéré, who commented on the high extinction coefficient (30). Dhéré realized that the purine and pyrimidine bases in nucleic acid are responsible for its ultraviolet absorption spectrum. His observations were made on yeast (ribose) nucleic acid. In 1931 Heyroth and Loofbourow found that thymus (desoxyribose) nucleic acid has the same ultraviolet absorption spectrum (43). It should not be supposed that an awareness of the spectroscopic properties of nucleic acids had become part of the common stock of knowledge in biochemistry before Caspersson entered the field. Quite the contrary; Dhéré's observations are not mentioned in Levene's book on nucleic acids, which means that they were far removed from the minds of active workers in this field. Caspersson was the first to recognize the great importance of spectroscopic measurements in the study of nucleic acids.

Shortly before Dhéré's paper appeared, Köhler published a description of the first quartz microscope, and, indeed, took with it microphotographs of chromosomes (51). The two advantages of the ultraviolet microscope over the ordinary microscope mentioned by Köhler are the increased resolving power due to the shorter wave length of light used, and, an even more significant advantage, that many objects are detectable in living cells because of naturally occurring differences in transmission of ultraviolet light, such differences being analogous to those brought about artificially by the staining techniques ordinarily used in microscopy. The latter advantage is well illustrated by Köhler's micro-

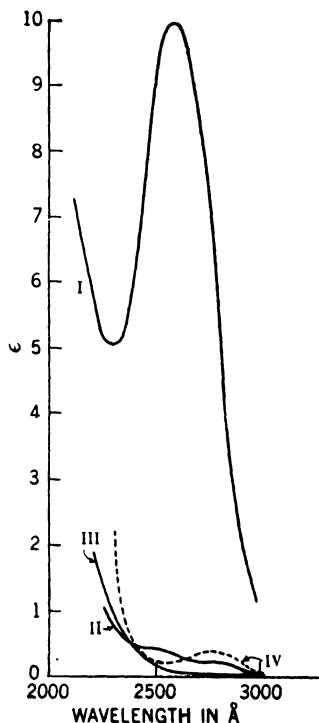


Fig. 1.—Absorption curves of  
I. 0.5% Sodium thymonucleinate;  
II. 0.5% Histone sulfate;  
III. 5% Protamine sulfate;  
IV. 0.5% Serum albumin (dotted) (Caspersson).

photographs of chromosomes. Köhler pointed out that chromatin absorbs ultraviolet light far more completely than does the surrounding protoplasm, so that his ultraviolet microphotographs closely resemble those taken with visible light of preparations stained with Heidenhain's hematoxylin.

Many years later (1928) Köhler again demonstrated the usefulness of the quartz microscope, emphasizing this time its value for cytochemical investigations (52). In a study of the cornea and lens of the eye he clearly showed how in microphotographs taken with monochromatic light the relative intensities of certain cell structures (the nucleus especially) vary with the wave length of the light used. As the monochromatic light passes up through the object on the slide, into the microscope and onto the photographic plate the relative densities observed on the plate are an indication of the relative quantities of light absorbed by each microscopic object on the slide. By comparing the relative densities at different wave lengths Köhler realized that the absorption spectrum of any given microscopic object on the slide can be obtained; and by comparing the band so obtained with the absorption spectra of purified, isolated substances placed before an ordinary quartz spectrograph it is possible to identify the chemical constitution of the objects in the microscopic preparation. As an example of the procedure, Köhler compared an absorption band lying between 2940 Å and 2570 Å which he observed in a microscopic preparation of the lens of the eye with a band that had been discovered in isolated lens proteins. The possibilities pointed out by Köhler were not taken advantage of immediately. Several years later (1931) a beautiful series of microphotographs of cells in various stages of mitosis were taken by Lucas and Stark (58), but it remained for Caspersson (1936) to demonstrate the full power, for cytochemistry, of the quartz microscope when used with monochromatic light of different wave lengths (14).

In Caspersson's hands, methods for the determination of the absorption spectra of cell structures have reached a high degree of sensitivity and precision. The apparatus used is still essentially the same as Köhler's, the important changes being the introduction of some new light sources and the use of photoelectric instead of photographic methods. Descriptions of apparatus and procedure are given in Caspersson's papers (16). Most of the advantages of making cytochemical observations *in situ* at the highest magnifications are obvious. What may not, perhaps, be apparent is that an enormous increase in sensitivity is gained. As little as  $10^{-11}$  mg. of protein or nucleic acid can be detected within a cell just because the absorbing structure is so much smaller than the microcuvette used in the usual spectrographic micro-methods. Caspersson's procedure can be used to study a number of chemical substances in the cell, but so far its most notable success has been with nucleic acids, and this is hardly surprising, considering their exceedingly intense absorption and their high concentration in the nucleus, and frequently in the cytoplasm as well.

Caspersson's results show that nucleic acids dominate the ultraviolet ab-

sorption in a chromosome to such an extent that the absorption spectrum of a metaphase chromosome is almost like that of pure nucleic acid (14).

Measurements on a dark band in one of the giant chromosomes of the salivary glands of a *Drosophila* larva also reveal the absorption spectrum of nucleic acid. Figure 2, curve 1, shows the absorption spectrum of such a chromosome fragment when compared with (curve 3) the absorption spectrum of a 10% solution of nucleic acid. It can be seen that the absorption curve of the chromosome is shifted slightly toward the longer wave lengths, due, presumably, to protein present in the chromosome segment. Curve 2 shows the absorption spectrum of a part of the same preparation near the edge of the cell. This curve shows the maximum, at 2800 Å, typical of proteins. The

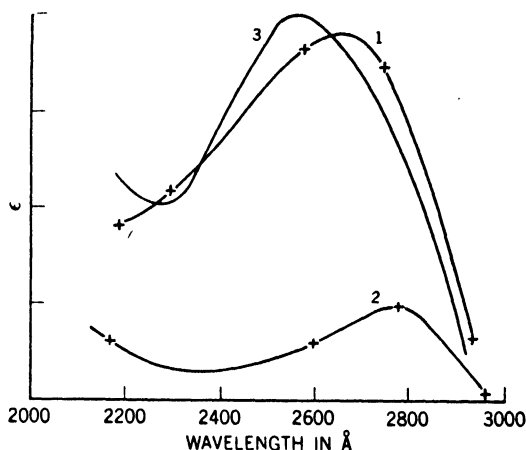


Fig. 2.—Absorption spectra of a chromosome fragment (Curve 1), of a region near the edge of the same cell (Curve 2), and of nucleic acid (Curve 3). (Caspersson (14).)

absolute height of curve 1, as well as the position of its maximum, shows that it is due to nucleic acid, for if it were due to protein, its height would require a layer of protein of 20% concentration and at least ten times as thick as the layer under observation. The concentration of nucleic acid in a chromosome is in the neighborhood of 10%.

After demonstrating that the dark-staining bands of the giant salivary gland chromosomes contain nucleic acid in high concentration, that the pale bands contain far less, and that both bands contain proteins, Caspersson proceeded to digest away some of the proteins. This was done with a trypsin solution to which lanthanum was added in order to precipitate the nucleic acid *in situ* while the protein to which it is attached is digested and washed away. As digestion proceeds and protein is washed away, it is as if a veil were removed, for it can now be seen that within the

dark bands there is a structure of more delicate bands that could not be seen before (14).

The quantity of nucleic acid in the nucleus varies under certain conditions. The variations observed by Caspersson had been noted by earlier investigators with the use of stains. The new observations are, however, far more convincing. Ultraviolet photographs of nuclei in cells of the digestive glands of *Helix pomatia* show that as secretion proceeds, the nuclei contain less and less nucleic acid. Apparently the nucleic acid in the nucleus takes part in the metabolic processes as work is done by the gland (14).

During cell division there are considerable changes in the nucleic acid content of the nucleus. Caspersson's observations on cells dividing in the course of spermatogenesis show that the quantity of nucleic acid increases in prophase and then decreases again in telophase (15).

Figure 3 contains the results of analyses on living cells as meiosis proceeds from early leptotene to diplotene. In Fig. 3,  $d$  is the diameter of the nucleus in  $\mu$ ,  $K$  is the extinction coefficient at 2570 Å and  $NS$  is the total amount of absorbing material in the cell, reckoned as nucleic acid. It is clear that the increase in amount of nucleic acid takes place very early, before the contraction of the chromosomes. The condensation of chromosomes is accordingly not directly due to the accumulation of nucleic acid.

Caspersson points out that the increase in nucleic acid occurs at the time that splitting of the chromosomes becomes apparent and this leads him to suggest that nucleic acid is connected with gene reproduction. Unfortunately cytogeneticists do not seem to agree about the time that gene reproduction occurs (48).

The results achieved by the technique of ultraviolet microscopy have been described without reference, so far, to the observations made with the Feulgen nucleal reaction. Results yielded by the two procedures concerning the presence of nucleic acid in chromosomes agree very satisfactorily. Agreement is obtained because deoxyribose nucleic acid is present. When a structure contains ribose nucleic acid instead, observations made by the two methods disagree, and yet supplement each other. By the ultraviolet absorption technique both ribose and deoxyribose nucleic acids are detectable and, indeed, by this technique alone it is impossible to distinguish between the two nucleic acids. This is because absorption in the ultraviolet depends on the purine and pyrimidine bases, and the difference between the two nucleic acids in this respect is very slight, one of the pyrimidines in deoxyribose nucleic acid being methyluracil (thymine) instead of uracil itself, as in ribose nucleic acid. The nucleal reaction, on the other hand, detects deoxyribose nucleic acid only. There is still, unfortu-

nately, no specific cytochemical test for ribose nucleic acid. At present the best indication that a structure contains ribose nucleic acid is that it has the absorption spectrum characteristic of a nucleic acid but that it fails to give a nucleal reaction; the inference, then, is that ribose nucleic acid is present. This is not conclusive evidence, for purines or pyrimidines in

Stage in meiosis	<i>d</i>	<i>K</i>	<i>NS</i> , 10 <sup>-3</sup> mg.
Early leptotene	13.1	0.456	19.6
	14.1	0.337	16.8
	13.5	0.390	17.8
	12.5	0.284	11.2
	12.9	0.398	16.6
Later leptotene to the beginning of pachytene	15.0	0.44	24.7
	10.6	0.796	22.4
	10.6	0.760	21.4
	13.0	0.585	25.5
	11.3	0.688	22.0
	18.1	0.300	24.3
	14.5	0.523	26.0
Zygotene	10.0	0.796	20
	12.8	0.568	23.3
Pachytene to the beginning of diplotene	12.0	0.699	25.2
	13.5	0.482	21.8
	11.9	0.658	23.4
	11.3	0.796	25.4
	13.5	0.585	26.5
	11.5	0.620	20.5
Diplotene	14.1	0.522	26.0
	15	0.509	28.4
	14	0.45	22.2
	17.5	0.347	26.5

Fig. 3.—Changes in nucleic acid content during meiosis.

considerable concentration might perhaps be present in some other form than as nucleic acid, and such is actually the case in striated muscle, in which the concentration of adeny nucleotides is so great that their precise localization in the muscle fiber can be determined, as Caspersson and Thorell have shown, by the use of the quartz microscope and ultraviolet light (23). A further test for ribose nucleic acid is provided by the enzyme, ribonuclease, isolated by Kunitz (54). If a structure in the cell appears to



contain ribose nucleic acid, in that it has the proper absorption spectrum and fails to give a nucleal reaction, the cytologist then applies Kunitz's ribonuclease. Disappearance of the characteristic absorption spectrum when reaction products are washed away is taken as confirmation of the presence (before treatment with the enzyme) of ribose nucleic acid. And yet even this evidence is not conclusive. Assuming that the enzyme is indeed pure and does not contain traces of other enzymes, it is still impossible to say that, although it acts on ribose nucleic acid, it acts on nothing else. Having mentioned these difficulties, the results of cytochemical tests for ribose nucleic acid will be mentioned without further reference to possible pitfalls.

Within the nucleus itself there is a structure that contains ribose nucleic acid and little, if any, desoxyribose nucleic acid. The nucleolus, as Caspersson and Schultz have shown, has an absorption spectrum indicating the presence of nucleic acid (22). Since nucleoli, with few exceptions, are known to be Feulgen-negative, it is inferred that they contain ribose nucleic acid. By observing the response to stains of the nucleolus before and after treatment with ribonuclease, Brachet has independently demonstrated that the nucleolus contains ribose nucleic acid (12). The evidence is now as complete as present technique permits, for Gersh has recently shown that the ultraviolet absorption band characteristic of nucleic acid in the nucleolus disappears after treatment with ribonuclease (35). It may be that some ribose nucleic acid is present in the chromosomes, along with the great quantities of desoxyribose nucleic acid known to be there, but at present there is no way of testing this possibility. The great accumulations of ribose nucleic acid, it will be seen later, are in the cytoplasm.

The intense absorption at  $2600 \text{ \AA}$  of nucleic acid is, of course, a great advantage in cytochemical study, but even when the absorption band of a substance is far less intense an analysis may be possible. In a recent study Caspersson has attempted to characterize the absorption bands of protein present in chromosomes and nucleoli, although the differences in absorption upon which the analysis is based are admittedly very slight (17, 18). It has already been seen (by comparing curves 2 and 3 in Fig. 2) that protein affects the absorption curve of a chromosome. Caspersson believes that from the precise nature of this protein effect, it is possible to tell whether the protein is of the histone type, containing a high percentage of basic amino acids, or whether it is of the same general type as serum albumin. The ability to distinguish spectroscopically between histones and less basic proteins depends upon what appears to be a definite, though small, difference in the ultraviolet absorption curves of serum albumin and of

histone prepared by acid extraction from the nucleohistone of the thymus gland. The histone curves shown by Caspersson unfortunately contain so much nucleic acid still adhering to the histone that the point of maximum absorption cannot be directly perceived. When the absorption due to this impurity is subtracted, it appears that the point of maximum absorption of the histone is at a slightly longer wave length than the maximum of serum albumin. Ultraviolet absorption curves of a number of different histones which have recently been made (67) do not show the difference between histone and albumin described by Caspersson. The histones were separated by acid extraction from nucleohistones prepared from a great variety of sources—thymus, spleen, pancreas, liver, sea-urchin sperm, shad sperm. In each case so little nucleic acid remained in the preparation that the position of maximum absorption was directly apparent, and in each case it was the same—at a slightly shorter wave length than that of egg albumin, which is 2800 Å. It seems doubtful whether Caspersson's "histone type of absorption" is in fact due to histone.

#### IV. Preparation and Properties of Constituent Parts of Chromosomes

In preparing the constituent parts of chromosomes there are two problems: the first is to make sure that the substances isolated are not derived from the cytoplasm; the second is to assure that the methods of isolation change the substances as little as possible. Miescher showed that the constituents he isolated were indeed derived from the nucleus and not from the cytoplasm by first separating the nucleus from the remainder of the cell. Since both the pus cells and sperm used by Miescher have but a scanty cytoplasm, the problem was not difficult. A preliminary isolation of the nucleus was also necessary when Kossel prepared histone from avian erythrocytes, but in this case too, separation of the nucleus was facilitated by the peculiar properties of the red cell. A method of separating nuclei applicable to plant and animal cells, in general, has been needed. The flotation technique devised by Behrens seems to be generally applicable (7). In this procedure the tissue is dried, pulverized, and then suspended in fluids of specific gravity chosen so that in them nuclear particles are separated, when centrifuged, from particles of the cytoplasm and cell membrane. Behrens has used this method to study the enzymes present in separated nuclear and cytoplasmic material (8). Another important use of the method was to isolate the nuclei of rye embryo cells and then prepare from them desoxyribose nucleic acid (33), which had previously been de-

tected in the nuclei of these cells by the nucleal reaction. A somewhat simpler method of isolating nuclei is to grind a tissue in 5% citric acid and then separate the nuclei by differential centrifugation (28, 59, 87).

The methods of extraction used by Miescher, Kossel and Levene were quite drastic. The very first step was the separation of nucleic acid and protein by strong acid and in later stages of the preparation strong alkali and high temperatures were used. It is now known that this rough treatment depolymerized the nucleic acid to a considerable extent. The earlier workers were hardly aware of the existence of high polymers and they were only occasionally troubled by suspicions of how they were degrading nucleic acid while isolating and purifying it. The fact is, however, that with their preparations they worked out many of the fundamental points in the organic chemistry of nucleic acid. The need for gentler treatment was felt when the biochemist inquired into the functions of nucleus and chromosomes.

A landmark in the movement to study nucleic acids in relatively intact form is the paper published by Einar Hammarsten in 1924 (39). From that time to the present, important contributions to the physicochemical investigation of nucleic acid have come from Hammarsten's group in Stockholm. For the preparation of nucleic acid Hammarsten went back to a method devised by Bang in 1904, which in the intervening twenty years does not appear to have been used (3). The curious thing is that before 1904 there were a number of workers (Lilienfeld, Huiskamp) who had discovered that the nucleohistone of the thymus can be prepared by a gentle procedure, simply extraction in a large volume of water (56, 47). Bang found that the nucleohistone prepared in this manner can be split into nucleic acid and histone by saturating with sodium chloride. Nucleic acid precipitates in fibrous form when alcohol is added to the salt solution. The nucleic acid so prepared readily dissolves in water to form a viscous solution.

The physicochemical properties of the desoxyribose nucleic acid prepared in this way have been studied by Hammarsten (40), Caspersson, Signer (82), Pedersen (88), Astbury (1), Greenstein (36) and their collaborators. The picture that emerges appears important for conceptions of chromosomes and genes. Desoxyribose nucleic acid is one of those highly polymerized, threadlike particles many of which are now familiar to chemists both as naturally occurring and synthetic products. That it is highly polymerized is shown by its failure to diffuse through a cellophane membrane and by its rate of sedimentation in the ultracentrifuge. The particles of nucleic acid in solution range in size from 200,000 to over 1,000,000.

Sedimentation experiments also indicate that the particles have a thread-like form, for the sedimentation rate varies markedly with the concentration of nucleic acid. The frictional ratio  $f/f_0$  is 2.5, indicating extremely asymmetric particles. The conclusions arrived at by sedimentation experiments are borne out by observations on the viscosity and double refraction of flow of desoxyribose nucleic acid in solution. The particles are optically negative; that is, their great polarizability is perpendicular to the longitudinal axis. Their strongly double refracting components appear to be arranged in a definite pattern. It is supposed that the purine and pyrimidine rings lie in planes perpendicular to the longitudinal axis of the molecule. This picture is confirmed by an x-ray study of the fibers. The strongest period along the fiber axis is at 3.34 Å, an interesting observation because this spacing is almost exactly equal to the distance between successive side chains in a fully extended polypeptide.

The highly polymerized, viscous, birefringent particles of desoxyribose nucleic acid are readily depolymerized and so lose their viscosity and birefringence. The existence of a depolymerase was recognized by Feulgen and Levene. Greenstein has made a careful study of thymonucleodepolymerase (37). He finds that extracts of many tissues and the sera of many animals possess this activity. In addition to the existence of what appears to be a depolymerizing enzyme, proteins show a marked tendency to depolymerize nucleic acid.

In the chromosomes nucleic acid occurs in association with protein. The proteins known to be derived from nuclei are those basic proteins, the histones, and protamines, which have been prepared, in most instances, from fish sperm. The method of preparation has, in almost every case, been essentially the same as the one used by Miescher when he discovered protamine by extracting it from salmon sperm with hydrochloric acid. In only one case, until recently, has the complex of nucleic acid and protein been extracted, as a whole, in relatively intact condition, and this is the nucleohistone of the thymus gland. A method has recently been introduced for preparing the nucleic acid protein complex of chromosomes, which seems to be quite generally applicable (67). To extract these nucleoproteins from the cell and to separate them from other cellular constituents nothing more drastic is used than neutral sodium chloride solutions of varying concentrations. A characteristic common to all these nucleoproteins is their solubility in 1 M NaCl and their insolubility in physiological saline. The insoluble material is highly fibrous, so that it is readily wound around a stirring rod. The nucleic acid present in these substances is desoxyribose nucleic acid and the protein is either a histone or protamine, depending on

the material from which it is prepared. Histones have been known to be constituents of certain cell nuclei for many years, but Kossel, the foremost investigator of histones, believed that they occur not in all nuclei, but only in the nuclei of certain kinds of tissues. It can now be seen that histones (or in some cases protamines) are probably as universal constituents of nuclei as is desoxyribose nucleic acid. Fibrous nucleohistones have been prepared from mammalian liver, kidney, spleen, brain, pancreas, thymus; from frog, trout, shad and sea urchin sperm, from the liver, spleen and blood cells of the dog fish, and from wheat germ. The nucleic acid content of these substances ranges from 31 to 66%.

These nucleoproteins resemble the nucleohistone prepared in the past from thymus glands by extraction with water. A difference between these fibrous materials and thymus nucleohistone as ordinarily prepared is that, when the latter is precipitated in physiological saline, the precipitate is not fibrous. It can be shown that its fibrous character is destroyed by the method of preparation. A nucleohistone which precipitates in fibrous form can be prepared from the thymus if extraction is accomplished with 1 *M* NaCl instead of with distilled water. This fibrous nucleohistone of the thymus is soluble in very dilute salt solutions (0.02 *M*) and is precipitated when the salt concentration is increased to 0.14 *M*, but the precipitate is no longer fibrous. Once the nucleohistone is dissolved in 0.02 *M* NaCl its fibrous character is lost. Nucleohistones extracted with 1 *M* NaCl from liver, spleen and other organs also lose their fibrous character once they have been dissolved in 0.02 *M* NaCl. Nucleohistone is ordinarily prepared from the thymus by extraction with approximately 0.02 *M* NaCl and this apparently is the reason the material does not precipitate in fibrous form. When the fibrous and the nonfibrous materials are both dissolved in 1 *M* NaCl certain differences between them become apparent: the fibrous material is far more viscous and exhibits a more marked streaming birefringence. Some of the fibrous nucleoproteins, those, for example, prepared from wheat germ and trout sperm, are insoluble in 0.02 *M* NaCl (67).

Although the nucleus was not isolated as a preliminary step in preparation of these nucleoproteins there can be little doubt that they are in fact derived from nucleus and not from cytoplasm. The first indication of nuclear origin is given by the resemblance between the chemical constitution of these substances and the constitution of substances known to be derived from the nucleus. Further evidence of nuclear origin is that in some instances, trout sperm for example, so much material is extracted that the scanty cytoplasm present in the cell could not possibly account for it. The

nucleus is approximately nine-tenths of the cell volume in trout sperm, and over 90% of the dry weight of a sperm suspension is extractable as desoxyribose nucleic acid and protamine. But the source of the nucleoprotein cannot be thus determined by calculation alone in extractions from the mammalian liver, in which the nucleus is somewhat less than one-tenth of the cell volume. In this case a direct cytological study of the effects of extraction of cells shows decisively that the nucleoprotein is derived from the nucleus (67). There are also indications that impurities derived from the cytoplasm are not present to any considerable extent.

The bond between nucleic acid and protein in the desoxyribose nucleohistone is loose and apparently of a saltlike nature. Bang's discovery that histone and nucleic acid in thymus nucleohistone can be separated simply by adding neutral sodium chloride in high concentration shows clearly the looseness of the bond. Nucleohistones prepared from thymus and other tissues can also be separated into their component parts by a procedure first used by Sevag (77), in which the nucleohistone solution is shaken with a chloroform-octyl alcohol mixture (67). In the course of prolonged shaking histone accumulates at the water-chloroform interface while nucleic acid remains in solution. The effectiveness of this separation is evidence for a loose bond between histone and nucleic acid.

The nature of the bond between protamine and nucleic acid can be shown in a different way. The fibrous complex extracted from trout sperm can be separated into protamine and nucleic acid simply by dissolving it in 1 *M* NaCl and dialyzing against 1 *M* NaCl. It is found that all the protamine passes through the cellophane membrane leaving practically pure nucleic acid behind. This experiment, incidentally, shows the small size of the protamine molecule, even when, in its preparation, no strong acid is used (67). Dialysis of the nucleoprotein against water (instead of against 1 *M* NaCl) precipitates the material in fibrous form.

The interaction between protein and desoxyribose nucleic acid is strikingly shown by the fact that the nucleoprotein precipitates in neutral 0.14 *M* NaCl. In this medium both histone and nucleic acid are soluble when taken separately. Mixing two such solutions of isolated histone and nucleic acid results in recombination of the two to form a precipitate. The saltlike nature of the complex formed becomes apparent when a protein such as serum albumin is used instead of histone. Serum albumin does not form a precipitate with nucleic acid in the neighborhood of neutrality, but when the medium is made more acid than pH 5 a precipitate appears. Serum albumin cannot form a saltlike union with nucleic acid until it is in a medium on the acid side of its isoelectric point, and the same is true of

histone, but, at neutrality, histone is in a medium far to the acid side of its isoelectric point, which is in the neighborhood of  $pH$  11.

Interaction between histone and nucleic acid is also shown by spreading experiments (67). Nucleic acid does not spread at an air-water interface. Histone spreads to give a film 7–9 Å thick, the thickness characteristic of other proteins. Nucleohistone forms a film 15–16 Å thick.

There is interaction between histone and nucleic acid even when the nucleohistone is in solution. Experiments in the Tiselius electrophoresis apparatus show that nucleohistones of thymus (38) and liver (67) migrate as negatively charged, electrically homogeneous single components in neutral solutions of low ionic strength. When a mixture of nucleic acid and serum albumin is placed in the Tiselius apparatus under the same conditions, two components are observed and yet some interaction between nucleic acid and serum albumin can be detected, even on the alkaline side of its isoelectric point (85).

Preparation of nucleoproteins from chromosomes of many different kinds of cells makes possible a comparison of these materials from several points of view. The cytologist has insisted on the lengthwise splitting of the chromosome into precisely equal halves at each cell division. The cytoplasm, on the other hand, is frequently observed to divide quite unequally, so that after a number of divisions, a marked cellular differentiation takes place. Differentiation of cytoplasm means that re-duplication of chromosomes in different cells of the same organism is accomplished in vastly different environments. The question arises: How uniform is the constitution of the chromosomes throughout the cells of a differentiated organism? As an answer to this problem it would be of interest to compare the various histones prepared from different tissues of the same organism with respect to their amino acid composition and immunological properties. Investigation along these lines has only just begun. In the course of preparing histones, cursory comparisons have been made, and the results so far obtained emphasize the resemblance between histones prepared from different tissues of the same organism (67). There are, indeed, certain uniformities in composition of histones prepared from the most diverse sources. They all contain tyrosine; some do, and others do not contain cystine; none of them contain tryptophane. The absence of tryptophane is striking, when it is considered that these histones were prepared from sources as biologically unrelated as the brain of the calf, shad sperm, sea-urchin sperm and wheat embryo. In his book on "The Protamines and Histones" (p. 84) Kossel lists thymus histone and the histones prepared from two varieties of fish sperm as giving color reactions for tryptophane.

Histone prepared from highly purified thymus nucleohistone does not give a reaction for tryptophane (67). It would be of interest to extract and purify the nucleohistones from the two kinds of fish sperm mentioned by Kossel and then test the histones for tryptophane.

Preparation of nucleohistones from different sources and the separation of the nucleic acids from these complexes make possible a comparison of the desoxyribose nucleic acids present in different tissues. Using the diphenylamine procedure of Dische (31) it is possible to compare the ratio of desoxyribose to phosphorus in various preparations and in this respect they all appear to be the same. The reaction with diphenylamine can also be used to determine the ratio of purines to pyrimidines in nucleic acid. By comparing the intensity of color formed when diphenylamine reacts with desoxyribose, with the intensities formed when it reacts with the purine and pyrimidine nucleosides of desoxyribose, it is found that all of the pentose in a purine nucleoside reacts with diphenylamine whereas almost none of the pentose in a pyrimidine nucleoside does. This difference, due to the much greater tendency of the purine than of the pyrimidine nucleoside to be hydrolyzed by the acid present in diphenylamine reagent, makes possible a determination of the ratio of purine to pyrimidine nucleosides in a desoxyribose nucleic acid. All of the nucleic acids so far considered, including those from animal sources as well as that from wheat germ, agree in having equimolar quantities of purines and pyrimidines—a definite restriction in possible variations among the desoxyribose nucleic acids (66).

## V. Nucleoproteins in Chromosomes

The desoxyribose nucleoproteins are isolated from chromosomes and their properties are investigated with the ultimate purpose of understanding what part they play as constituents of the chromosomes. Although this knowledge is, of course, still fragmentary, it is worth considering. First of all comes the curious solubility of the desoxyribose nucleoproteins. They are all insoluble, within the neighborhood of neutrality, in physiological saline, and yet many of them have the unusual property of being soluble in salt solutions that are either more concentrated or less concentrated than physiological saline. In the chromosome they must, therefore, be insoluble. The insolubility of these nucleoproteins appears to be correlated with the salt concentration of the body fluids. This correlation is strikingly shown when a preparation is made from an elasmobranch fish, such as the dog-fish. The osmotic pressure of the body fluids of this fish are equivalent



to a sodium chloride solution that is 0.50 *M*. The nucleoprotein prepared from dog-fish is insoluble in 0.50 *M* sodium chloride (67). And yet nucleoproteins prepared from animals such as the calf or trout (for which physiological saline is 0.14 *M*) are soluble in 0.50 *M* sodium chloride, but insoluble in 0.14 *M* NaCl.

The physical properties of chromosomes must be due in large measure to the tendency of desoxyribose nucleic acid to polymerize and so form highly asymmetric particles. It was at first thought that, in the chromosome, these particles are markedly oriented (80), but it now appears that the degree of birefringence observed was due to the use of fixatives and other agents. Using dichroism in the ultraviolet as a sensitive indicator of orientation, Caspersson has recently shown that in giant salivary gland chromosomes of *Drosophila* the orientation of nucleoprotein molecules is slight, if indeed they are oriented at all (17a). Caspersson suggests that orientation of nucleoprotein probably occurs, but on such a small scale that it cannot be detected at present. He points out that a nucleic acid chain 0.02  $\mu$  long, which would be far below the resolving power of the microscope, could align a polypeptide chain with a molecular weight of several thousands.

The chromosome possesses a continuous protein framework the integrity of which does not depend upon presence of nucleic acid. This was shown in a most elegant manner by Mazia, who used a nuclease prepared from the spleen to digest away the nucleic acid of salivary gland chromosomes of *Drosophila* (61). The chromosomes are still clearly visible after nuclease treatment and they still stain with the ninhydrin protein reagent. The removal of nucleic acid is demonstrated by failure of the chromosomes after nuclease treatment to give a Feulgen nucleal reaction and by their failure to stand out clearly when photographed with ultraviolet light.

Mazia considers the continuous protein framework of a chromosome to be composed of histone (60). This conclusion is based on experiments with proteolytic enzymes. When a chromosome is treated with trypsin it is completely disintegrated. With pepsin, on the other hand, the integrity of the chromosome is maintained, although the volume shrinks considerably. Histones are digested to a considerable extent by trypsin while they are merely broken down to "histopeptones" by pepsin. The shrinkage of the chromosome in pepsin is attributed by Mazia to presence of a "matrix" protein with many acidic groups which is digested by pepsin. It seems to the writer that the shrinkage could also be explained by the breakdown of histone to histopeptone. If a matrix protein with acidic groups should prove to be present, it may well be attached to the structure of the chromosome by forming saltlike linkages with the basic groups of histone. Com-

pounds of basic proteins with other proteins are well known. An example is the protamine-insulin compound used in the treatment of diabetes.

If histone is considered to form a continuous fibrous structure in the chromosome, the question arises as to whether the protamines of fish sperm chromosomes also form a continuous structure. The known properties of protamines do not seem to fit them for this role. There is so little other

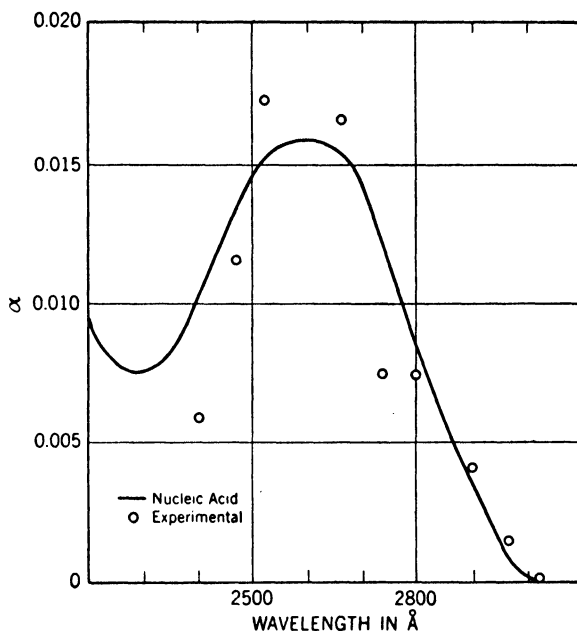


Fig. 4.—Experimental values of  $\alpha$  (ordinate) are plotted against the wavelength (abscissa) for comparison with the absorption spectrum of thymonucleic acid (Caspersson, 1936), the latter being shown as a full curve. (Stadler and Uber (84)).

protein present in the sperm head of salmon and trout that it is puzzling to know what protein in this case provides a continuous structure for the chromosome.

The chemical constitution of the chromosome is gradually being elucidated. The chemical nature of the gene is a far more difficult problem. The great accumulation of desoxyribose nucleoproteins in the chromosome strongly suggests that these substances either are the genes themselves or are intimately related to genes. The close relation between nucleic acid

and genes is also shown by experiments on the genetic effects of ultraviolet radiation. Stadler and Uber have studied the relative genetic effectiveness of monochromatic ultraviolet light of various wave lengths on maize (84). A great difficulty in the investigation is the determination of the effective energy at the chromosome because in radiating the pollen grain some energy is absorbed before it reaches its target in the chromosome.

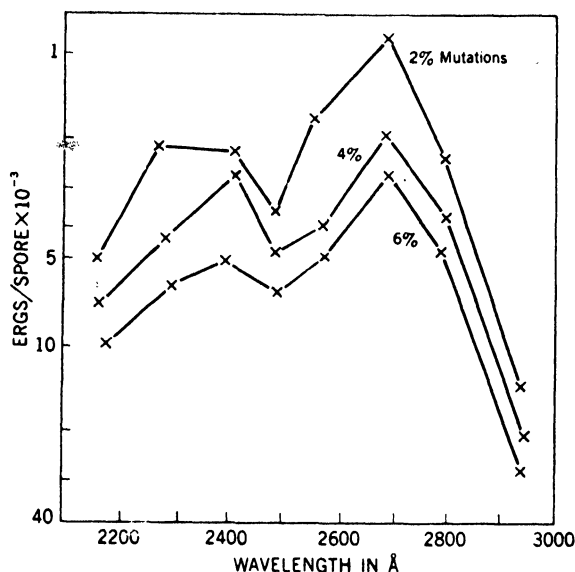


Fig. 5.—Reciprocal of relative energy at eight wave lengths for 2, 4 and 6 per cent mutations. (Hollaender and Emmons (46).)

In Fig. 4 the relative effectiveness of radiation at each wave length is plotted against the wave length, and the points so obtained are compared with the absorption spectrum of nucleic acid. "Considering the various approximations involved, the agreement is surprisingly good and lends further support to the frequently advanced hypothesis that nucleic acid is intimately associated with the functioning of the germinal material" (84).

The effect of ultraviolet radiation can be studied more precisely on micro-organisms than on maize. The complication that arises is that in the fungus that has been most carefully investigated the variants produced by irradiation are not well understood genetically. As a result of experiments on the fungus *Trichophyton mentagrophytes*, Hollaender and Emmons have plotted the reciprocal of the energy required to produce a definite percentage of

mutations against the wave length of the radiation used, Fig. 5. The maximum at 2650 Å coincides with the high absorption coefficient of nucleic acids near this wave length (46).

The kind of experiment that is needed to place the chemistry of the gene on a firm basis is one in which substances extracted from the chromosomes of an organism are administered to a mutant form of the same organism which suffers from a deficiency in its germinal material. This has been the method of endocrinology; a substance extracted from a gland is administered to an organism suffering from a "deficiency" of this gland. If genes are indeed distinct and separable chemical substances, the circumstances under which this procedure can be followed may perhaps be discovered.

## VI. Cytoplasmic Nucleoproteins

It is now known that desoxyribose nucleic acid occurs in the cell nucleus and not in the cytoplasm and that nuclear-like bodies containing desoxyribose nucleic acid are present in yeast and bacteria as well as in the cells of higher plants and animals. Ribose nucleic acid, on the other hand, is the nucleic acid of cytoplasm in both animals and plants although smaller quantities exist in the nucleolus and its presence in other parts of the nucleus cannot at present be excluded.

Until recently it was supposed that desoxyribose nucleic acid is present in the nuclei of animal cells only and that ribose nucleic acid is in the nuclei of plant cells. Bacteria and yeast were supposed to be without nuclei. And yet the presence of ribose nucleic acid in cells of animals was discovered many years ago by O. Hammarsten (41). It is rather surprising to find that the problem of its localization in the cell does not seem to have been mentioned by the earlier workers. The first mention of the problem of localization appears in 1924 in Feulgen's paper on the nucleal reaction (34). When Feulgen discovered desoxyribose nucleic acid in the nuclei of plant cells, he suggested that ribose nucleic acid is located in the cytoplasm. He showed that the procedure followed by Osborne (71) and his colleagues in extracting ribose nucleic acid from wheat germ does not affect the nuclei of wheat germ cells, and he suggested that the marked basophilic character of the cytoplasm of the cells is due to the presence of large quantities of ribose nucleic acid.

Definite proof of the presence of ribose nucleic acid in cytoplasm came in 1938 when Behrens and Mahdihassan (in Feulgen's laboratory) separated the cytoplasm from the nuclei of rye embryo cells by Behrens' flotation method. From the separated nuclei desoxyribose nucleic acid had already

been isolated (33). The next step was to isolate ribose nucleic acid from the cytoplasmic moiety (6). The isolated ribose nucleic acid amounted to 3% of the separated cytoplasm.

A similar distribution of desoxyribose and ribose nucleic acid in the nucleus and cytoplasm, respectively, of yeast has been demonstrated by Delaporte using less direct methods (29). Washing yeast with water or a dilute bicarbonate solution removes the volutin granules of the cytoplasm and from the washings ribose nucleic acid can be isolated. This extraction leaves the Feulgen-positive nuclei unaffected. When the cells are subsequently extracted with stronger alkali the Feulgen-positive granules in the cell vanish and desoxyribose nucleic acid can be isolated from the extract. The ribose nucleic acid content of yeast determined by extraction and isolation can amount to as much as 5 to 10% of the dry substance.

The amounts of ribose nucleic acid present in cells are surprisingly large. In some bacteria ribose nucleic acid accounts for as much as 15 to 20% of their dry weight (79). The pancreas contains more ribose nucleic acid than any other animal tissue does; 10% of its dry weight consists of this nucleic acid (49). These amounts are of course small compared with the 60% of desoxyribose nucleic acid present in the sperm heads of some fish but they show clearly that ribose nucleic acid is, considered purely quantitatively, an important constituent of cytoplasm.

Recent investigation emphasizes the relation between the basophilic character of cytoplasm and its ribose nucleic acid content. Evidence for such a correlation was provided many years ago. In 1913 Van Herwerden treated a number of different cells with a nuclease prepared from spleen and found that as a result of nuclease activity the basophilic properties of certain cytoplasmic granules disappeared (89). The effect of nuclease was interpreted as demonstrating the presence of nucleic acid in the basophilic granules. No distinction was made between the two kinds of nucleic acid. More recently Brachet has made a systematic study of basophilic cytoplasmic structures using a nuclease known to act on ribose nucleic acid and not on desoxyribose nucleic acid (12). Structures which stain intensely with pyronin lose this property after treatment with ribonuclease. In this way Brachet has shown that, in general, cells that are strongly basophilic owe this property to the presence of ribose nucleic acid.

The distribution of ribose nucleic acid demonstrated by the use of stains and ribonuclease is also demonstrated by ultraviolet microspectrophotometry. The great advantage of the latter method is its quantitative nature. Caspersson and his colleagues have carried out an extensive series of investigations on cytoplasm of many different cells. This survey of cyto-

plasm began with a study by Caspersson and Schultz of growing tissues (21). They compared the cytoplasm of rapidly growing cells with that of more mature cells in the tissues of *Drosophila* larvae and in the root-tips

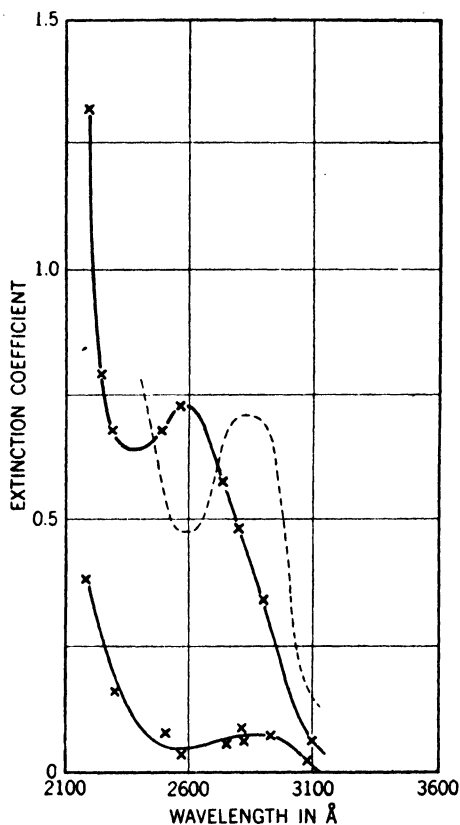


Fig. 6.—The ultraviolet absorption spectra of the cytoplasm of a cell from the growth zone (upper curve) and from the base (lower curve). In the dotted curve the values of the lower curve are multiplied by the factor 8 to permit a comparison of shapes. (Caspersson and Schultz (21).)

of plants. They found that where mitoses are occurring there is a high concentration of absorbing materials with a spectrum of the nucleic acid type. The difference in cytoplasmic absorption spectrum between cells of

a root-tip which are dividing and others which will no longer divide is shown in Fig. 6. The nondividing cells have a far lower extinction coefficient and a position of maximum absorption characteristic not of nucleic acid but of protein. At the end of their paper describing this investigation Caspersson and Schultz note that, "It seems likely that a high concentration of nucleic acids is the basis of the generally noted basophilia of embryonic tissues." This prediction has now been borne out by Caspersson and Thorell in a thorough investigation of the chick embryo. Especially high concentrations of nucleic acid are present in the cytoplasm of cells in the earliest stages of development when the rate of growth is most intense (24).

A similar investigation has been made of yeast, comparing rapidly growing cells with other cells (13, 19). In yeast it is possible to show that a high concentration of nucleic acid in the cytoplasm is correlated with growth and not merely with metabolic activity, for by controlling the composition of the medium in which the yeast cells are suspended, *it is possible to have a culture that is fermenting rapidly but not growing*. In such an active but stationary culture the total cytoplasmic nucleic acid is at a relatively low level, though the volutin granules can be observed to be rich in nucleic acid. When growth occurs a striking increase in ultraviolet absorption due to nucleic acid is observed.

Caspersson considers that since the synthesis of protein is one of the main events occurring in rapidly dividing cells, it is most likely that the presence of high concentrations of ribose nucleic acid in the cytoplasm during growth is in some way concerned with protein synthesis. Protein synthesis apart from growth takes place on a grand scale in certain glands. It has been estimated that in 24 hours the pancreas produces an amount of protein equivalent to as much as 20% of its dry weight. The basophilic cytoplasm of many of these gland cells, notably those in the pancreas, have long been recognized. Caspersson and his colleagues find that in the pancreas, parotid and other glands, the secretions of which contain large quantities of protein, the cytoplasm is rich in nucleic acid (20). When the pancreas is stimulated to intense secretory activity by injection of pilocarpin an increase in ribose nucleic acid content occurs, and it can be seen that this is especially marked in the region of the nuclear membrane. The accumulation of ribose nucleic acid in this region had already been noted by Caspersson and Schultz in the sea-urchin egg, and it was suggested that synthesis of nucleic acid takes place at the nuclear membrane (22).

The proteins associated with desoxyribose nucleic acid in the nuclei of both animal and plant cells are the histones and protamines. It is not

known whether the proteins associated with ribose nucleic acids form as distinct a group as the basic proteins of the nucleus. The protein moiety of a ribose nucleoprotein would not be expected to be as basic as a histone because whereas a nucleohistone contains about 40% of nucleic acid, a ribose nucleoprotein usually contains far less nucleic acid, in the neighborhood of 10%. Very little is known about the proteins of the cytoplasmic nucleoproteins.

The type of bond between protein and nucleic acid is different in the ribose nucleoproteins from what it is in the desoxyribose nucleoproteins. In the latter the linkage appears to be primarily of a saltlike nature. The bond between nucleic acid and protein in a naturally occurring ribose nucleoprotein is nonpolar. It is readily broken by some of the procedures that have frequently been used to prepare these nucleoproteins. Just what the bond is remains obscure, but it certainly is different from the saltlike linkage formed when the separated components of a ribose nucleoprotein are brought together (42, 78).

Some of the cytoplasmic nucleoproteins exist in the cell in the form of granules. The volutin granules of yeast have already been mentioned as containing ribose nucleic acid. It has been stated by Menke that chloroplasts contain ribose nucleic acid (63). Claude has recently shown that cytoplasmic granules containing nucleoproteins and lipid can be isolated from cells by differential centrifugation (25). Granules were isolated from mammalian tissues, chicken tumor and yeast. From liver two definite fractions were obtained. In one fraction the particles were relatively large, ranging in size between 0.5 and 3  $\mu$ . The other fraction consists of much smaller particles, 60 to 200  $m\mu$ . Both fractions contain nucleoprotein. The small granules are considered to be mitochondria or fragments of mitochondria. In them ribose nucleic acid represents as much as 10 to 15% of the nucleoprotein portion of the particle. Claude has shown that physical and chemical agents readily liberate nucleic acid from these granules, and he points out that in cytological preparations the basophilia of cytoplasm may be due to nucleic acid that has diffused away from injured mitochondria.

In this connection the relations between nucleic acid and the volutin granules of yeast are of interest, especially since the observations of Caspersson and Brandt were made not on cytological preparations but on living yeast cells by means of ultraviolet light and a quartz microscope (19, 13). In resting cells volutin granules containing nucleic acid are present in the hyaloplasm, which absorbs ultraviolet light only feebly. As the cells begin to grow the granules swell, multiply and gradually disappear.



At the same time the hyaloplasm itself absorbs more and more ultraviolet light and finally appears rather homogeneous. Observations of the cells in the dark field also show that as the hyaloplasm becomes opaque to ultraviolet light the granules disappear.

## VII. Interrelations

In this paper the nucleoproteins of nucleus and cytoplasm have been discussed separately. It can hardly be supposed that they actually exist in the cell isolated from each other. The first indication of a metabolic relationship between the two nucleic acids was discovered by Brachet (10). It was shown by him that in the unfertilized sea-urchin egg there is far more ribose than desoxyribose nucleic acid, but that as the egg develops after fertilization the desoxyribose nucleic acid content rises and at the same time the quantity of ribose nucleic acid falls. To explain these observations Brachet suggested that one nucleic acid is synthesized at the expense of the other. Brachet's results were adversely criticized by Blanchard because in his preparations of the two nucleic acids from unfertilized sea-urchin eggs approximately equal amounts were isolated (9). Brachet's results were, however, borne out by the ultraviolet absorption measurements, made directly on the sea-urchin egg by Caspersson and Schultz (22). Their absorption data show that the major part of the nucleic acids in the unfertilized sea-urchin egg is Feulgen-negative.

Since Brachet's work, relationships between the nucleic acids of a far-reaching nature have been envisaged. The relationships involve three regions of the cell: a part of the chromosome known as *heterochromatin*, the nucleolus and the cytoplasm. The heterochromatin is that part of the chromosome which remains visible during the period in the mitotic cycle when the chromosome as a whole becomes lost to sight. Now the visibility of a chromosome whether by staining or by ultraviolet photography depends primarily on its nucleic acid content. Heterochromatin may therefore be characterized as that portion of a chromosome which retains its high content of nucleic acid in the interphase when the rest of the chromosome (the so-called *euchromatin*) loses much of its nucleic acid. Heterochromatin has been considered to be genetically inert; no genes have been located in it, and it has made little observable difference in an organism whether a heterochromatic region of a chromosome were altogether absent or present in excess. And yet it has been known for some time that the properties of the nucleolus are associated with certain heterochromatic regions. The nucleolus, in turn, appears to be connected in some way with

the quantity of ribose nucleic acid in the cytoplasm, and it should be remembered that the nucleolus itself contains ribose nucleic acid. It seems probable that heterochromatin, though lacking in genes, is not inert but serves as a regulator of nucleic acid metabolism both in the nucleus and cytoplasm. Heterochromatin, according to this view, is inert only in the sense that it is nonspecific in its activity. It has an over-all influence due to its control over the production of nucleic acids. The new conception of the role of heterochromatin first appears in the work of Caspersson and Schultz (81). Much has been added both experimentally and speculatively by Darlington (28). The most recent contribution to heterochromatin comes from Painter and Taylor (72). They find that in the toad, heterochromatin is segregated for the most part into discrete granules entirely removed from the chromosomes and, though separate, this chromatin still appears to function.

### VIII. Viruses

The viruses that are well defined chemically are the crystalline plant viruses. They have been compared with genes for the following reasons: because they are self-duplicating bodies, submicroscopic in size; because they mutate; and because they are nucleoproteins. Some properties of viruses that are of interest in the study of other nucleoproteins will now be discussed.

The plant viruses that have been isolated are all ribose nucleoproteins. In this respect they differ from most of the nucleoproteins in the cell nucleus, and for this reason they should perhaps be compared with those self-duplicating plastids, the chloroplasts, in the cytoplasm of many plants. It has been reported that chloroplasts contain ribose nucleic acid (63). A comparison of plastids and the plant viruses both from a chemical and an evolutionary point of view might be significant. Unfortunately very little is known about the chemical constitution of chloroplasts. When the self-duplicating nature of chloroplasts is considered their lamellar structure appears to be significant. An ultraviolet microphotograph of a chloroplast taken by Köhler and published by Menke shows the lamellar structure beautifully (64).

There are, of course, many viruses in addition to those that have been crystallized, and in one of them, vaccinia, it has been definitely shown that desoxyribose nucleic acid is present (62, 83). At least 5.6% of the dry substance of this virus is desoxyribose nucleic acid (45). This is far less than the quantity present in chromosomes. It is comparable to the quanti-

ties present in some bacteria. In streptococci 2 to 6% of the dry weight is desoxyribose nucleic acid (79). In these bacteria this represents only 10 to 30% of the total nucleic acid present, the remainder being ribose nucleic acid. Little, if any, ribose nucleic acid was found in vaccinia. It is possible that in the washing to which vaccinia particles are subjected preparatory to analysis much ribose nucleic acid is removed. Washing a yeast culture removes most of its ribose nucleic acid (without extracting the desoxyribose nucleic acid present), and yet after this treatment the cells grow when placed in a suitable medium (29).

In the bacteriophage contradictory results concerning the presence of nucleic acid have been reported (70, 50). It is worth noting, however, that in a carefully prepared and highly purified sample of phage material, a positive Feulgen reaction was obtained (76). This material had a phosphorus content of 3.7% (75). It would be of great interest to know if these observations mean that the material contained approximately 40% of desoxyribose nucleic acid—the quantity present in many nucleohistones (67).

Concerning the ribose nucleic acid of the crystalline plant viruses there is some highly interesting information, the equivalent of which is lacking for the ribose nucleic acids of animals and plants. Loring has made a careful comparison of the ribose nucleic acid prepared from tobacco mosaic virus with that prepared from yeast (57). These two ribose nucleic acids apparently differ with respect to one of their pyrimidine nucleotides—uridylic acid. The brucine salts of the two uridylic acids differ in their optical activity and solubility. Since they agree in their elementary composition, they are probably isomers. It would be interesting to know whether such differences occur in other ribose and desoxyribose nucleic acids. The specificity of a nucleoprotein may reside in its nucleic acid as well as in its protein moiety.

Ribose nucleic acid as ordinarily prepared is not nearly as highly polymerized as is undegraded desoxyribose nucleic acid. The highly polymerized desoxyribose nucleic acid particles are markedly asymmetric. In making these comparisons it is important to recognize that fairly strong alkali is usually employed in the preparation of ribose nucleic acid and that if a similar procedure is followed in preparing desoxyribose nucleic acid, it also is depolymerized to a considerable extent. It has recently been shown that if alkali is not used in the isolation of ribose nucleic acid, at least in one instance a preparation with highly polymerized asymmetric particles is obtained.

Cohen and Stanley have prepared ribose nucleic acid from tobacco mosaic

virus by a method which avoids the use of alkali (26). In the new procedure a solution of the virus is heated at  $100^{\circ}$  for one minute. As a result of the heat denaturation, the bond between nucleic acid and protein is broken, and most of the nucleic acid remains in the supernatant fluid when the protein coagulum is centrifuged.

This nucleic acid is far more highly polymerized than is the nucleic acid prepared from tobacco mosaic virus by treatment with alkali. The highly polymerized material has an average particle weight of 300,000. The particles are highly asymmetric. They depolymerize spontaneously to form asymmetric particles having a molecular weight of approximately 61,000. These particles are broken down by alkali to still smaller particles having a molecular weight of 15,000 and an axial ratio of 10. It is possible that end to end polymerization of the 15,000 units forms the 61,000 units and that these in turn polymerize end to end to form the 300,000 units. On the basis of certain assumptions, it can be calculated that the 61,000 units are about 700 Å long and the 300,000 about 3000 Å long. The virus molecule itself is 2800 Å long and 150 Å wide. Either of the nucleic acid particles that have not been treated with alkali is too long to be able to lie across the virus molecule. Cohen and Stanley point out that the long threadlike nucleic acid particles having a molecular weight of 300,000 have a length which appears to be that of the virus molecule.

Plant viruses, like animal viruses, occur in numerous strains and there can be little doubt that new strains are constantly arising. When a new strain appears it is difficult to determine whether a mutation has just occurred or whether the mutation had taken place at some previous time and has become apparent due to factors of selection. If two strains are present in a virus-stock a mutation must have taken place at some time, for it can be regarded as certain that the viruses grouped together as strains have arisen from a common source.

The differences in general chemical properties between viruses of closely related strains are not marked and yet in some cases a mixture of two strains can be separated by means of their differences in filterability. Differences have also been detected in the immunological properties of related strains. Plant viruses are found to be antigenic when injected into animals, and anti-sera for closely related strains may be produced. It is then found that although related strains react strongly with each other's sera, they are not serologically identical. Each strain contains a number of antigens; the more closely related two strains are, the more antigens they possess in common, and yet each strain also possesses specific antigens.

The immunological differences found in related strains of virus are

usually attributed to differences in the protein components of the virus nucleoproteins, though differences in the nucleic acids have not been excluded. Recent investigation shows decisively that there are differences in the amino acid constitution of closely related virus strains (53). These results form a background for considerations concerning the nature of mutations in the self-duplicating particles of bacterial, plant and animal cells.

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# EFFECTS OF TEMPERATURE ON ENZYME KINETICS

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## I. Introduction

The effects of temperature upon enzyme systems have been of interest almost since enzymes were discovered over a century ago. Despite this fact it is only within the past twenty years that major advances have been made in interpreting these phenomena. The development of this field has been retarded by serious problems encountered in enzyme kinetics (41). Advancement was facilitated by drawing a distinction between the two major effects of temperature: (a) that on the enzyme-catalyzed re-



action, and (b) that on the inactivation of the enzyme. Both chemists and biologists have contributed to the study of temperature effects on enzymes; the former have attempted to treat the subject as a problem in inorganic catalysis, while the latter have thought in terms of the properties of living organisms, endowed with all the complexities of physiological systems. Modern enzymologists, making use of both points of view, have attempted to integrate the field and to present a plausible interpretation of the effects of temperature on enzyme systems, although it must be realized that the major problems are as yet unsolved.

## II. Chemical Kinetics as a Function of Temperature

In 1889 Arrhenius (1) examined the available data on chemical kinetics as a function of temperature and proposed the following equation as one best fitting the data

$$\frac{d \ln k}{dt} = \frac{A}{RT^2} \quad (1)$$

where  $k$  is the rate,  $R$  the gas constant,  $T$  the absolute temperature,  $t$  is °C. and  $A^*$  a constant which has subsequently been called the activation energy. Integration of this equation yields

$$\ln \frac{k_2}{k_1} = \frac{A}{R} \left( \frac{1}{T_1} - \frac{1}{T_2} \right) \quad (2)$$

from which it is obvious that  $A$  can be calculated from the rates at two different temperatures or from the slope of the straight line when  $\log k$  is plotted against  $1/T$  ( $A = \text{slope} \div 4.58$ ). Equation (1) may also be integrated and written in the form

$$k = Ce^{-A/RT} \quad (3)$$

where  $C$  is a constant. The quantity  $A$  was originally identified by Arrhenius as a sort of reaction energy for the conversion of normal, inactive molecules into reactive ones. The quantity  $C$  turns out to be, or at least to contain, a "frequency factor" which is primarily concerned with the probability with which active molecules (*i. e.*, those which have acquired the necessary activation energy) pass over into products (2).

While originally an empirical expression for the change of reaction rate with temperature, the equation soon received confirmation on theoretical grounds, since it can be derived from the van't Hoff equation which de-

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\* In subsequent work Arrhenius and many biologists have used the symbol  $\mu$  instead of  $A$ , while chemists have largely employed the symbol  $E$  or  $\Delta H^*$ .

scribes in a similar way the change in the equilibrium constant,  $K$ , with temperature,

$$\frac{d \ln K}{dt} = \frac{\Delta H}{R\bar{T}^2} \quad (4)$$

where  $\Delta H$  is the heat of reaction, and is the algebraic sum of the activation energies of the forward and reverse reactions. More recently the Arrhenius equation has been derived in essentially the same form from the collision theory of chemical reactions by Hinshelwood (3) and Moelwyn-Hughes (4). The most rigorous development of a theoretical equation relating kinetics to temperature has come from statistical mechanics where the equation derived is similar to, but not identical with, the Arrhenius equation (*cf.* Glasstone, Laidler and Eyring, 5).

### 1. Interpretation of the Mechanism of Chemical Reactions

Chemical reactions including catalytic ones, it is generally agreed, are associated with an energy of activation representing the minimum energy the system must acquire before it can undergo the appropriate change. Only those molecules which have the minimum requirement of energy (*i. e.*, the activation energy) will react. From considerations of the distribution of molecular velocities obtained from the Boltzman equation the fraction of the molecules having an energy of  $E$  or greater is  $e^{-E/RT}$ , or

$$\frac{n^*}{n} = e^{-E/RT} \quad (5)$$

where  $n^*$  is the number of activated molecules and  $n$  is the total number of molecules (6).

The mechanism by which activated molecules are formed is not entirely clear, but as a first approximation can be related to a collision frequency in bimolecular and to a vibration energy in unimolecular reactions. These factors are represented numerically by the constant  $C$  in equation (3). Temperature changes the proportion of active molecules greatly. For example, an increase of ten degrees at  $27^\circ$  increases the average translational energy  $10/(273 + 27)$  or only 3.3%. It increases the number of rapidly moving molecules very appreciably, however, and in this way accounts for the typical high temperature coefficient ( $Q_{10}$ ) for chemical reactions of 2 to 3. The fraction of molecules having an energy of  $E$  or greater is  $e^{-E/RT}$ ; assuming  $E = 25,000$  cal. per mole, a rise in temperature from  $300^\circ$  to  $310^\circ$  abs. causes the fraction of molecules having energies of 25,000 cal. or more to jump from  $5.49 \times 10^{-10}$  to  $20.9 \times 10^{-10}$ . It is apparent that a  $10^\circ$  change in temperature has increased by 380% the number of molecules having an energy greater than 25,000 cal. (6).

The activated molecule or complex not only has a higher energy content than the average molecule but has this extra energy localized, at least in part, at a particular valence bond at which cleavage subsequently occurs. The time required for this locali-

zation of energy could account for the time-lag between activation and decomposition. In certain reactions this time-lag, which is of the same order of magnitude as the period of revolution of an electron in its orbit, may involve the collecting by successive collisions of sufficient energy at a critical point in the molecule to produce an unstable activated complex. The activated complex like other molecules possesses the usual distribution of translational and rotational states, and all but one of the expected normal modes of vibration. The missing normal mode of vibration has become an intramolecular translation. This represents motion of the parts of the activated complex relative to one another and occurs at the point where the activated complex subsequently decomposes.

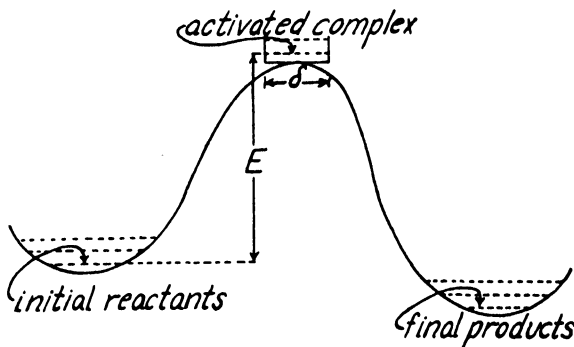


Fig. 1.—Schematic representation of the mechanism of reaction. In order to form the final products the initial reactants must pass over the energy barrier by acquiring the activation energy  $E$ , thereby becoming an activated complex.

(From Laidler and Eyring (7).)

The mechanism of activation can be visualized by a consideration of Fig. 1. Before the initial reactants can be converted into the final products the reacting system must "surmount an energy barrier" by acquiring the activation energy  $E$  which is represented by the difference in energy between the initial state and the maximum of the curve, the top of the barrier.

"From the point of view of its extra degree of translational freedom which corresponds to its decomposition along the abscissa of the figure the activated complex is regarded as being a free particle in a one dimensional box of length  $\delta$  . . . ." (Laidler and Eyring, 7).

In the activated molecule the length of a bond is increased by about 10% of its normal value; and the energy of activation has been found to be approximately one-fourth the sum of the energies of the bonds involved in the reaction. The energy of activation is considerably less than the

energy required to break the bonds in the reacting molecules, because new bonds are formed in the activated state. Hence the energy of activation is the difference between the energy obtained by the formation of these bonds and the amount necessary to break the bonds in the reactants (5). Of the many different pathways by which initial reactants may be converted into final products the reaction will proceed by the one involving the least energy of activation, and this will be least when a minimum number of bonds are broken and formed (6).

## 2. Modified Forms of the Arrhenius Equation

The quantity  $C$  in equation (3) may be thought of as a "frequency factor" which gives a measure of the rate at which active molecules (*i. e.*, those having acquired the necessary activation energy) are converted into reaction products. In bimolecular reactions where activation may result from collision the rate becomes

$$k = Ze^{-E/RT} \quad (6)$$

where  $Z$  is the number of collisions per second between reacting molecular species. The same equation applies to unimolecular reactions where  $Z$  is roughly the vibration frequency of atoms in the molecule. Although this equation accurately predicts the rates of many reactions, it by no means describes all of them. It has therefore become necessary to introduce the constant  $P$  into the equation

$$k = PZe^{-E/RT} \quad (7)$$

where  $P$  is a factor essentially independent of temperature representing the unknown conditions such as steric relationships which must be fulfilled. It seems reasonable that in many reactions there may be collisions of molecules with ample energy which do not react at the moment of collision because of unfavorable orientation of the molecules or other circumstances (8). Hinshelwood (3) and Moelwyn-Hughes (4) have been especially responsible for demonstrating the wide applicability of the above equation in interpreting chemical kinetics.

Both La Mer (9) and Rodebush (10) have pointed out that the collision theory is only an approximation and is not thermodynamically correct, and that actually both  $PZ$  and  $E$  of equation (7) will vary slightly with temperature. Theoretical derivations based on statistical mechanics by these workers as well as by Eyring and associates (5) have led to a statistical theory of reaction rates which can be expressed in the equation

$$k = k_1 \frac{KT}{h} e^{\Delta S/R} e^{-E/RT} \quad (8)$$

where  $k_1$  is the transmission coefficient which can usually be assumed to be unity,  $K$  is Boltzmann's constant,  $h$  is Planck's constant,  $\Delta S$  is the entropy of activation in calories per degree per mole, and the other symbols have their usual meaning. The entropy of activation normally changes little with temperature and is independent of the energy of activation; its value is determined by the general nature of the activated complex and is very sensitive to quantum effects. In a typical reaction it has a small positive value (often about 10 cal./degree) and any large departure from this indicates an activated complex in which new degrees of freedom are active (8) (*cf.* section on enzyme inactivation by heat). If it be assumed that the reacting molecules are rigid spheres, the statistical and collision theories give the same results, and so the collision theory may be considered a special case of, or a first approximation to, the statistical theory (8).

### 3. *Kinetics in Heterogeneous Systems*

In heterogeneous reactions involving the adsorption of reactants upon the surface of a catalyst, the reacting species require activation by collision or otherwise just as in homogeneous reactions. As would be expected, the Arrhenius equation is satisfied, for there seems to be no fundamental difference between a catalyzed and an uncatalyzed reaction. The catalyst constitutes an additional reactant and, as such, it is incorporated in the activated complex. The catalyst will in general lower the energy of activation of the reaction and this lowering is perhaps the most important factor in catalysis (3). Since the velocity of surface reactions increases exponentially with temperature in accordance with the Arrhenius equation (11), the adsorbate can be regarded as an Arrhenius intermediate, the decomposition of which will normally determine the kinetics and activation energy of the over-all reaction (12). The typical heterogeneous reaction will involve the following stages: diffusion to the catalytic surface, adsorption, reaction on the surface, desorption of the products, followed by diffusion from the catalytic surface (5). Each of these stages follows the Arrhenius equation, but in such a series the over-all rate is determined by the rate of passage of activated complexes over the highest energy barrier. It is thus possible to disregard all aspects of the reaction other than the equilibrium between the initial state and the rate-determining activated state. Diffusion may be the slow step ("pacemaker") in a heterogeneous reaction if the activation energy is very low (roughly 3000 cal. per mole), and adsorption or desorption the slow step when the activation energy changes with the relative concentrations of catalyst and substrate and also changes with temperature.\* In the typical reaction, however,

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\* This is not true in the case of a reaction on a saturated surface (a zero order reaction) where the rate is independent of concentration and temperature within fairly wide limits (11).

the chemical reaction which occurs on the catalytic surface will determine the over-all rate and activation energy. In enzyme reactions, if the same principles of chemical kinetics hold true, this rate-determining step will normally be the cleavage of the enzyme-substrate complex into enzyme plus reaction products.

### III. The Inactivation of Enzymes by Heat

Enzymologists have long realized that enzyme reactions increase in rate with temperature and reach a maximum at the "optimum temperature." Above the optimum the rate decreases with further rise in temperature. This optimum temperature has been used to characterize a particular enzyme, but it is now generally realized that the optimum temperature is not a constant for a given enzyme, since it varies widely with such factors as enzyme and substrate purity, presence of activators and inhibitors, and the method used in measuring the rate of the catalyzed reaction. Tamman in 1895 (13) correctly explained the optimum temperature of enzyme reactions by suggesting that two independent processes were simultaneously accelerated by temperature: the catalyzed reaction and the thermal inactivation of the enzyme. By assuming the temperature coefficient of inactivation to be greater than that for the reaction, the effects of temperature can be explained. At temperatures lower than the optimum it is the catalyzed reaction that is chiefly affected, while at temperatures higher than the optimum the inactivation of the enzyme by heat is the predominant factor.

As soon as the two effects of temperature upon enzyme-catalyzed reactions were recognized, the phenomena were studied separately. The effect upon inactivation will be considered first and the effect upon the rate of the enzyme-catalyzed reaction will be dealt with in a subsequent section.

The inactivation of enzymes by heat was investigated in the absence of substrate; the amount of active enzyme remaining after a given exposure to a certain temperature was determined by cooling to a standard temperature and adding substrate. Temperature inactivation may become appreciable at a temperature as low as 30° and for the majority of enzymes is very marked at 50–60°. The misconception in arbitrarily choosing 37.5° as a working temperature in enzyme studies is obvious in view of the partial inactivation of some enzymes at this temperature.

The inactivation of enzymes by heat is usually unimolecular and increases exponentially with temperature in accordance with the Arrhenius equation over a fairly wide range, indicating that the activation energy

is independent of temperature changes. From the data presented in Table I it is apparent that the activation energies for enzyme inactivation by heat are high, usually between 40,000 and 100,000 cal. per mole. On the basis of the collision theory of chemical reactions (3), those with such

TABLE I  
ENERGIES AND ENTROPIES OF INACTIVATION OF ENZYMES

Enzyme	$\mu$ , cal./mole	$\Delta S$ , cal./degree/mole	pH
Pancreatic lipase (16)*	46,000	68.2	6.0
Trypsin (16)	40,800	44.7	6.5
	67,600 ( $\Delta H^*$ )	...	..
Entero-kinase (16)	42,800	52.8	6.5
Trypsin-kinase (16)	44,900	57.6	6.5
Pancreatic proteinase (16)	38,000	40.6	..
Pepsin (15)	55,600†	113.3	6.4
Pepsin (15)	67,470†	135.9	5.7
Milk peroxidase (15)	185,300†	466.4	..
Rennen (15)	89,350†	208.1	..
Malt amylase (15)	41,630†	52.3	..
Invertase (15)	52,350†	84.7	5.7
Invertase (15)	86,350†	185	5.2
Invertase (15)	110,350†	262.5	4.0
Invertase (15)	74,350†	152.4	3.0
Emulsin (15)	44,930†	65.3	..
Leucosin (15)	84,300†	185	6.1
Insulin (15)	35,600†	23.8	1.5
Goat hemolysin (15)	198,000†	537	..
Vibriolysin (15)	127,950†	326	..
Tetanolysin (15)	172,650†	459	..
Pancreatic amylase (27)	39,100	...	6.5
Pancreatic amylase (27)	41,000	...	7.0
Mussel catalase (28)	10,000	...	7.0
Beef catalase (30)	45,000	...	..
Kidney catalase (33)	40,000	...	7.0
Hemoglobin (30)	60,000	...	..
Papain (29)	75,000	...	7.0
Solanin (29)	62,000	...	7.0
Asclepain <i>m</i> (29)	76,000	...	7.0
Asclepain <i>s</i> (29)	25,000	...	7.0
Bromelin (29)	46,000	...	7.0
Luciferase (31, 94, 95)	55,000	184	7.3
Mold kinase (32)	53,500	...	4.8

\* References to the literature are given in parentheses.

†  $\Delta H^*$  not  $\mu$ ,  $\mu = \Delta H^* + RT$ .

high activation energies should proceed with zero velocities at normal temperatures, yet enzyme inactivation occurs rapidly below 100°. Moelwyn-Hughes (14) has explained this discrepancy by assuming 10–20 internal degrees of freedom in the enzyme molecule. A more elegant explanation has been offered by Eyring and Stearn (15) who interpret the rapid rate of enzyme inactivation as related to the high entropy of activation. The entropies of activation in cal./mole/degree have been calculated for the inactivation of certain enzymes from the statistical theory of reaction rates (equation (8)) and are presented in Table I. It is apparent that the very high entropy increases of the order of magnitude of 100 cal./degree, which occur on enzyme inactivation, counterbalance the large activation energies and result in rapid reaction rates instead of the infinitely slow ones predicted without reference to entropy changes (16).

#### *Relationship of Heat Inactivation of Enzymes to Protein Denaturation*

The similarity between the high values for the activation energies of enzyme destruction and protein denaturation has been pointed out by many workers, and has been interpreted as evidence for the protein nature of enzymes and for the identity of the mechanisms of heat denaturation of proteins and heat inactivation of enzymes. This theory gained in plausibility with the demonstration that all crystalline enzymes which have been isolated are proteins. The theory was proved to be correct for certain crystalline proteases by Northrop and co-workers (17). With such crystalline enzymes as pepsin, trypsin and chymotrypsin the inactivation of the enzyme by heat (as measured by the loss in activity) is exactly paralleled by the disappearance of dissolved native protein from solution and the precipitation of heat-denatured protein. Such enzyme inactivation and denaturation is partially reversible on cooling for pepsin and chymotrypsin, and completely reversible for trypsin, if heating has not been too prolonged. For trypsin the equilibrium between active and inactive (denatured) enzyme shifts with temperature and is characterized by a heat of reaction ( $\Delta H$  in equation (4)) of 67,000 cal./mole, while the activation energy is 40,160 cal./mole (16, 18, 24). The reverse reaction, the regeneration of active trypsin, should thus have a negative heat of reaction of  $40,160 - 67,600 = -27,440$  cal. and on the basis of collision theory alone should proceed extremely rapidly. The theory of reaction rate based on statistical mechanics also takes into account the high loss in entropy of  $-168.4$  cal./degree/mole and leads to the correct value of the rate (16). Luciferase also appears to be an enzyme capable of reversible



inactivation by heat with an activation energy of 55,000 cal. (31). Pressure has a retarding action on the denaturation of luciferase (94, 95).

Recent work indicates that not only are energies of activation similar for enzyme inactivation and protein denaturation, but also that entropies of activation fall within the same range for both (5). The high entropies of activation are considered indicative of an activated complex in which new degrees of freedom become active and suggest that enzyme inactivation (denaturation) is essentially a dissociation process (19). More light has been cast upon the nature of denaturation by Steinhart's (20) study of the inactivation of crystalline pepsin. The rate of destruction of pepsin increases in proportion to the fifth power of the hydroxyl ion concentration; this is evidence that the rate is proportional to the concentration of a pepsin ion in which all five of the primary amino groups have undergone acidic dissociation. The over-all energy of activation is 63,500 cal. of which 45,200 cal. is the heat of dissociation of 5 amino groups (*i. e.*, 9040 cal. for the heat of dissociation of a single proton) and 18,300 cal. represents the energy quantity involved in the final activation process according to La Mer (21). Eyring and Stearn (15) have pointed out that the enormous entropy of 136 cal. per degree for pepsin inactivation is almost solely associated with a loss of structure or increase in randomness during the ionization process; little change in entropy is involved in the final step of activation leading to denaturation. It appears that the first steps in activation leading to denaturation involve the breaking apart of salt bridges between acid and basic groups held together by hydrogen bonds according to Mirsky and Pauling (22). The final step in activation leading to denaturation lies in the breaking of one or more bridges which are not destroyed by ionization, such as a covalent cystine bridge (15).

In view of the role of ionization in the inactivation of pepsin, the energy and entropy of activation would be expected to vary with the *pH* of the solution. This is true for many enzymes where  $\mu$  and  $\Delta S$  are a maximum at the *pH* of maximum stability of the protein and fall off on either side (5, 16, 34). A change in activation energy with *pH* is by no means universal, however, for Pace (23, 24, 25) has reported that  $\mu$  for the denaturation of certain proteases is essentially constant over a considerable *pH* range. Neither  $\mu$  nor  $\Delta S$  for enzyme inactivation changes significantly with temperature (16). The energy of activation is much higher for crude preparations of enzymes than for highly purified ones, but no explanation for this is available (14). When the denaturing agent is not heat, a high temperature coefficient may not occur. For example, Gates (26) found that  $Q_{10}$  for the inactivation of crystalline pepsin by ultraviolet irradiation

(where the action is presumably on such amino acids as tyrosine, tryptophane and phenylalanine) is only 1.021, a value to be expected for a photochemical reaction.

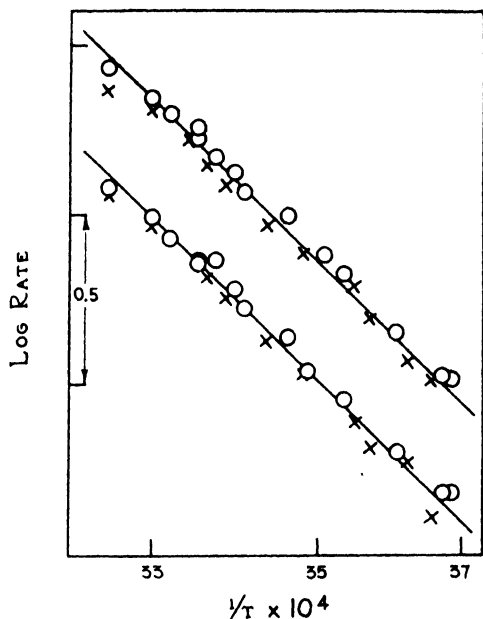


Fig. 2.—Curves for the inversion of sucrose catalyzed by yeast invertase showing the typical linear relationship between log rate and  $1/T$ .

Upper curve: Rate expressed as mg. invert sugar/min.

Lower curve: Rate expressed as monomolecular velocity constants.

For both curves  $\mu = 11,000$  cal. from  $0-35^\circ$ .

Above  $35^\circ$  the points fall off from the curve due to heat inactivation of the enzyme.

(From Sizer (80).)

#### IV. Kinetics of Enzyme-Catalyzed Reactions as a Function of Temperature

##### 1. Review of Early Work

At temperatures below the optimum the rate of enzyme reactions increases with temperature so that a rise of  $10^\circ$  effects an increase in rate by a factor of 1.3–3.5. It has been universally observed that the  $Q_{10}$  decreases with the rise in temperature and

TABLE II  
ACTIVATION ENERGIES OF ENZYME SYSTEMS\*

Enzyme	References	Substrates	pH	Temperature range, °C.	Activation energy, cal./mole
Bone phosphatase	42	Sodium $\beta$ -glycerophosphate	9.1	12-42	9,940
Urease	47, 48, 49	Urea	7.0	0-45	8,700 or 11,700
Bacterial dehydrogenase	56	Acetate, glycine, glutamate, lactate, succinate, glucose, mannitol, galactose, xylose, sucrose	7.4	0-40	19,400
Bacterial dehydrogenase	56	Fructose, sorbose	7.4	0-40	19,400 or 25,000
Bacterial dehydrogenase	56	Pyruvate	7.4	0-40	25,000
Bacterial dehydrogenase	56	Formate	7.4	0-40	15,000
Tyrosinase	57	Maltose	7.4	0-40	21,200
Succinic dehydrogenase (bacterial)	55	Cresol, catechol	6.2	2-30	2,700
Muscle hydrogenase	79	Succinate	7.6	25-50	16,700
Muscle hydrogenase	79	Oxalacetate	7.0	20-40	5,200†
Dehydrogenase-cytochrome-cytochrome oxidase	52	Pyruvate	7.0	20-40	5,600†
Dehydrogenase-cytochrome-cytochrome oxidase	53	Succinate	7.4	20-40	11,200
Dehydrogenase-cytochrome-cytochrome oxidase	53	Succinate plus poisons	7.4	20-40	16,000, 17,500, 18,600 or 22,300
Dehydrogenase-cytochrome-cytochrome oxidase	53	<i>p</i> -Phenylenediamine	7.4	15-37	9,500
Cytochrome reductase	82	Dihydrotriphosphopyridine nucleotide	...	...	10,000
Pancreatic lipase	58	Tributylin	8.1	0-50	7,600
Pancreatic lipase	58	Tributylin	8.1	-70-0	37,000
Pancreatic lipase	60	Tributylin trivalerin, tricaproin, triheptylin, tricapylin	8.0	0-40	8,700
Trypsin	58	Casein	8.1	0-40	15,400
Trypsin	58	Casein	8.1	-70-0	65,000
Trypsin	59	Benzoyl-L-arginine amide	7.8	6-37	14,900
Trypsin	59	Chymotrypsin	7.5	0-20	16,300
Trypsin	59	Sturin	7.5	13-34	11,800
Chymotrypsin	59	Benzoyltyrosylglycyl amide	7.5	4-36	10,500

TABLE II (Continued)

Enzyme	References	Substrates	pH	Temperature range, °C.	Activation energy, cal./mole
Chymotrypsin	59	Pepsin (denatured)	7.5	13-35	11,200
Chymotrypsin	77	Casein	7.7	- 6-30	12,000
Catalase	84	Hydrogen peroxide	...	0-20	6,400
Yeast invertase	58	Sucrose	4.5	0-40	11,000
Yeast invertase	80, 43	Sucrose, raffinose	5.2	0-40	11,000†
Yeast invertase	58	Sucrose	4.5	-70-0	60,000
Malt invertase	43	Sucrose	4.8	2-35	13,000
Purine-aldehyde dehydrogenase (milk)	51	Acetaldehyde, benzaldehyde, xanthine, piperonal, acrolein	6.2	0-60	16,000
Purine-aldehyde dehydrogenase (milk)	51	Cinnamic aldehyde, <i>p</i> -methyl benzaldehyde, <i>o</i> -methoxy benzaldehyde	6.2	0-60	21,000
Purine-aldehyde dehydrogenase (milk)	51	Hypoxanthine	6.2	0-60	13,000
Purine-aldehyde dehydrogenase (milk)	51	Furfural, <i>p</i> -methoxy benzaldehyde	6.2	0-60	18,000
Luciferase (bacterial)	31	Luciferin	...	0-20	17,000
Esterase (grasshopper)	85	Methyl butyrate	...	0-35	5,700
Choline esterase	86	Acetylcholine	...	25-40	5,100
Tropine esterase	87	Atropine	8.4	25-38	12,000
Carbonic anhydrase	64	Carbon dioxide	7.4	1-13	8,900
Fat oxidase	83	<i>Lupinus</i> oil	...	5-35	11,700
Pancreatic amylase	88	Starch	...	20-40	12,500
Malt amylase	89	Starch	5.5	10-30	12,300
Root peroxidase	90	Hydrogen peroxide-leuco-malechite green	...	0-30	11,100†
Triose phosphate mutase	91	Triose phosphate-arsenate	7.5	22-38	16,700
Zymohexase	92	Hexose diphosphate	7.3	10-40	16,500†
Pepsin	77	Casein	...	- 1-30	17,700†

\* The activation energies in this table are limited to those systems which follow the Arrhenius equation over a wide temperature range.  $\mu$  values calculated from data obtained at only two temperatures are not included.

†  $\mu$  value calculated by reviewer from published data.

‡  $\mu$  value corrected for effects of mutarotation.

this decrease has been considered by many a characteristic peculiar to enzyme reactions, although Arrhenius and others have pointed out that a fall in  $Q_{10}$  with temperature is expected on theoretical grounds and is obtained experimentally for chemical reactions. Calculations have been made of temperature characteristics ( $\mu$  in the Arrhenius equation) from enzyme data in the older literature and the general conclusion has been drawn that enzyme reactions do not follow the Arrhenius equation (35, 36, 37). In reviewing the subject, Moelwyn-Hughes (14, 38) pointed out that enzyme-catalyzed reactions constitute the notable exception to the rule that chemical reactions follow this equation. Tabulations of early work on activation energies will be found in the follow-

TABLE III

ENERGIES AND ENTROPIES OF ACTIVATION FOR THE HYDROLYSIS OF PROTEINS AND PEPTIDES BY DIFFERENT CATALYSTS (FROM BUTLER, 59)

Catalyst	Substrate	Log <sub>10</sub> $k$ (0°)	$\Delta H^*$	$\Delta S$
Trypsin	Benzoyl-L-arginine amide	0.40	14,900	- 6.2
Trypsin	Chymotrypsinogen	2.6	16,300	+ 8.5
Trypsin	Sturin	3.33	11,800	- 4.7
Chymotrypsin	Benzoyltyrosylglycyl amide	1.57	10,500	-17.4
Chymotrypsin	Pepsin	2.34	11,200	-11.5
(Hydrogen ion)	Acetyl glycine	-6.47 (60°)	21,200	-24.8

$$^1 \Delta H^* = \mu - RT.$$

ing references: (16, 30, 35, 36, 37, 38, 39, 40). It should be pointed out, however, that much of this early work is unreliable because of reasons such as the following:

- (a) Lack of quantitative methods.
- (b) Failure to regulate pH and other factors, especially in work done previous to 1910.
- (c) Failure to avoid temperature inactivation of the enzyme at higher temperatures.
- (d) Improper calculation of rates. This, as Bodansky (41) has emphasized, is a serious objection to many studies on enzymes. The custom of using as a measure of rate the amount of reaction products produced in a given time at different temperatures is invalid in many cases where the kinetics change during the course of a single experiment (43).
- (e) Calculation of  $\mu$  from  $Q_{10}$ . A more accurate and reliable method is to calculate  $\mu$  from the slope of the line when log rate is plotted against  $1/T$ .

## 2. Review of Recent Work

Much of the recent work on enzyme kinetics as a function of temperature indicates, in contrast with early work, that many enzyme reactions follow the Arrhenius equation over a wide temperature range up to temperatures where heat inactivation of the enzyme becomes apparent. A typical example, the inversion of sucrose by invertase, is shown in Fig. 2. It is

apparent that the reaction follows the Arrhenius equation accurately from 0–35° with an energy of activation of 11,000 cal. per mole. Compilations of enzyme reactions which follow the Arrhenius equation over a wide range of temperature are presented in Tables II, III and IV.

Not only do enzyme reactions follow the Arrhenius equation above 0° but below this temperature as well (Fig. 3). A sharp break occurs in the relationship at about 0° for lipase, trypsin and pepsin, with very high activation energies characterizing these systems below and lower energies

TABLE IV  
ENERGIES OF ACTIVATION FOR THE HYDROLYSIS OF THE SAME SUBSTRATE BY ENZYMES  
FROM DIFFERENT SPECIES

Enzyme	Source	Substrate	$\mu$ , cal./mole
Invertase (80)*	Yeast	Sucrose	11,000
Invertase (43)	Malt	Sucrose	13,000
Bone phosphatase (42)	Man	Na $\beta$ -glycerophosphate	9,940
Bone phosphatase (42)	Cat	Na $\beta$ -glycerophosphate	9,940
Tyrosinase (57)	Mealworm	Catechol or cresol	2,700
Tyrosinase (57)	Mushroom	Catechol or cresol	2,700
Tyrosinase (57)	Potato	Catechol or cresol	2,700
Urease (48)	Jack bean	Urea	8,700 or 11,700
Urease (47)	Soy bean	Urea	8,700 or 11,700
Urease (49)	<i>Proteus vulgaris</i>	Urea	8,700 or 11,700
Luciferase (31)	<i>Photobacterium phosphoreum</i>	Luciferin	17,000
Luciferase (31)	<i>Vibrio phosphorescens</i>	Luciferin	17,000
Amylase (88)	Pancreas (hog)	Starch	12,500
Amylase (89)	Malt	Starch	12,300

\* References to the literature are given in parentheses.

characterizing these systems above this temperature (58) (*cf.* also 75). Such a break in the relationship at 0° might be expected in view of the changes indicated by Nord (69), which take place in the physical and colloidal properties of a system, when it passes from the liquid to the solid state. It is generally agreed that enzymes are not inactivated by storage at temperatures as low as –186° (58, 75, 77).

The  $\mu$  values obtained, with the exception of tyrosinase, are within the range expected for chemical reactions, but are lower than the values for the same reactions accelerated by inorganic catalysts. This agrees with the

theory that the more efficient the catalyst the lower the activation energy, since enzymes are more efficient than inorganic catalysts. The value of 2700 cal. for tyrosinase is in the range of values for diffusion, although Gould\* has suggested that a photochemical reaction may account for the low value. It is doubtful whether adsorption is the limiting factor in any of these reactions (11). Since enzyme reactions probably proceed by the formation of an enzyme-substrate intermediate (44).

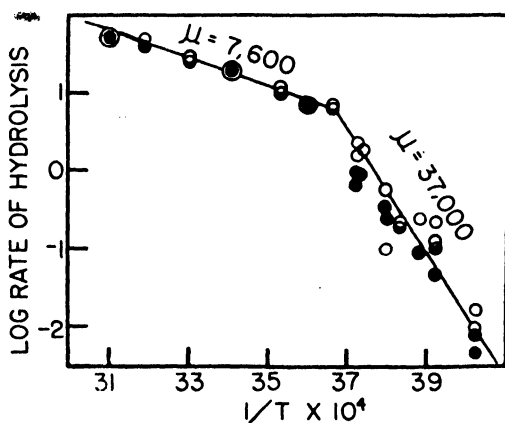
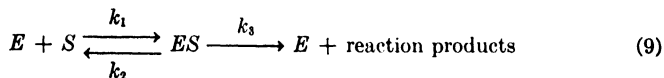


Fig. 3.—Log rate of hydrolysis of tributyrin by pancreatic lipase is plotted against  $1/T$ . A sharp break in the curve is apparent at  $0^\circ$ ; above  $0^\circ$   $\mu = 7,600$  cal., below  $0^\circ$   $\mu = 37,000$  cal. Above  $50^\circ$  heat denaturation of the enzyme occurs.

○ Digest contains 36.5 per cent glycerol

● Digest contains no glycerol.

(From Sizer and Josephson (58).)

the question arises as to which one of these reactions is characterized by the measured activation energy. Recent work by Chance (46) indicates that at least for peroxidase the breakdown of the enzyme-substrate complex is the slowest step; and thus  $\mu$  is a measure of the energy required to convert the complex into an activated molecule (*cf.* 59). Indirect evidence indicates a similar situation for many other enzyme systems.

\* Personal communication.

### 3. Effects of Various Factors upon the Activation Energy

The activation energy of an enzyme system theoretically should be independent of different environmental changes unless these factors alter the catalytic surface of the enzyme. For yeast invertase the activation energy was found by Sizer (43) to be essentially unaffected by (a) changes

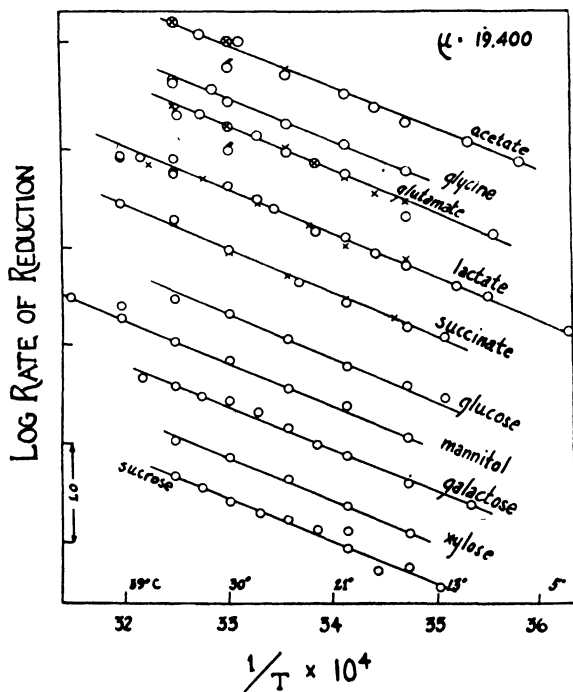


Fig. 4.—An Arrhenius temperature plot of the data for the dehydrogenation (as measured by the anaerobic reduction of methylene blue) of various substrates by *Escherichia coli* dehydrogenase.

(From Gould and Sizer (56).)

in pH from 3.2 to 7.9 (similarly for catalase  $\mu$  is independent of pH (78)), (b) changes in the electrolyte concentration, and (c) changes in the concentration of enzyme or substrate. The purity of the enzyme plays no apparent role in determining the activation energy, since identical  $\mu$  values were obtained for crude and partially purified aldehyde-purine dehydrogenase of milk (51), for soybean urease in different stages of purification



(47), and since impure and crystalline jack bean urease yielded the same energy of activation (47). Similarly, the presence of 36.5% glycerol in the digest does not affect the activation energy of pancreatic lipase (58).

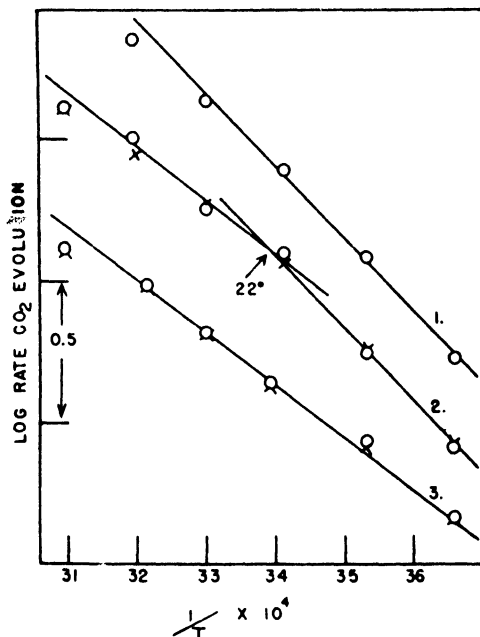


Fig. 5.—An Arrhenius plot of the effects of temperature upon the jack bean urease-urea system. In a digest containing mild oxidants  $\mu = 11,700$  cal. over the whole temperature range (curve 1). In a digest containing reductants  $\mu = 8,700$  cal. at all temperatures below the inactivation point (curve 3), but at intermediary oxidation-reduction potentials the system is unstable, and is characterized by energies of activation of 11,700 cal. below, and 8,700 cal. above the critical temperature of 22° (curve 2).

(From Sizer (48).)

The activation energy is not independent of all reagents. It would be expected that those which modify the catalytic surface would change the activation energy as well. Such appears to be the case for jack bean, soy bean and *Proteus vulgaris* ureases (47, 48, 49) for which the activation

energy may be either 8700 or 11,700 cal. (Fig. 5), depending upon the presence of either oxidizing or reducing agents which are known to oxidize or reduce sulfur linkages in the enzyme molecule (50) and thus modify the enzyme activity (93). A similar situation appears to hold true for the aldehyde-purine dehydrogenase of milk (51). In the oxidation of succinate by the succinic dehydrogenase-cytochrome-cytochrome oxidase system the addition of cyanide or other poisons causes the  $\mu$  value to shift (52, 53) (cf. Fig. 6). In such a system involving several steps, the inhibitor may retard only one step permitting it to become the slowest or rate-determining link upon which the activation energy depends.

Not only may the activation energy of an enzyme system be changed by the addition of an oxidizing or a reducing agent to the digest, but when the enzyme is in a metastable condition the activation energy may change suddenly at a critical temperature from one value to another. This point may be illustrated with jack bean urease, Fig. 5, where in the presence of mild reductants  $\mu = 8700$  cal. (curve 3) and with oxidants  $\mu = 11,700$  cal. (curve 1), while at intermediate oxidation-reduction potentials (about 440 mv. for crystalline urease)  $\mu = 11,700$  below and 8700 cal. above 22° (curve 2). A similar critical temperature with different activation energies above and below this point has also been encountered by Sizer (47, 49) for soy bean and *Proteus vulgaris* ureases and for the dehydrogenation of certain aldehydes and purines by the aldehyde-purine dehydrogenase of milk (51) as well as for enzyme action in the liquid and solid state (58). It seems possible that some of the earlier workers who reported a change in  $\mu$  with temperature may have been dealing with a similar situation, where one value characterizes the system below and another characterizes the system above a critical temperature. No adequate explanation of such a sharp transition from one activation energy to another at a critical temperature has been offered, but it might be suggested that this transition corresponds to a shift in the enzyme molecule from one configuration to another.

#### 4. Relationship of the Activation Energy to the Nature of the Substrate

In inorganic catalysis the energy of activation is frequently characteristic of the catalyst when similar substrates are used. For example, the hydrolysis of aliphatic esters catalyzed by  $\text{OH}^-$  and the hydrolysis of aliphatic acid amides by  $\text{H}^+$  are characterized respectively by  $\mu$  values of 11,000 and 20,000 cal. (54) (cf. also 3, 4). The activation energy of many reactions catalyzed by iron is about 16,200 cal. (55). For unrelated sub-

strates this generalization may not apply, *e. g.*, for sucrose hydrolysis catalyzed by  $H^+$   $\mu = 25,700$  not 20,000 cal.

This problem of the relationship of activation energy to the nature of the substrate was investigated by Sizer (43) with yeast invertase where the evidence indicated that this enzyme probably hydrolyzed raffinose as well as sucrose. In agreement with the foregoing the same activation energy was obtained for both substrates (*cf.* Table II). Similar results

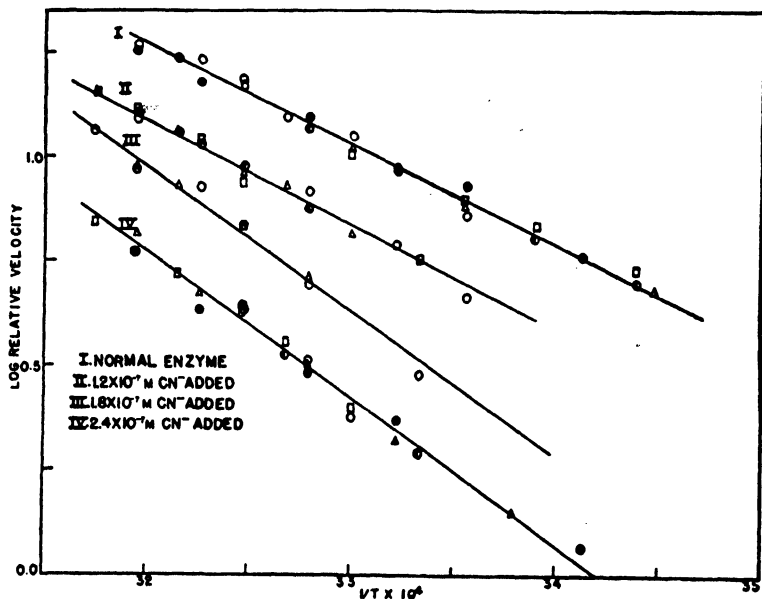


Fig. 6.—An Arrhenius equation plot of oxygen consumption by the succinate-succinic dehydrogenase-cytochrome-cytochrome oxidase system of beef heart. The addition of a low concentration of cyanide does not affect the  $\mu$  value, while higher concentrations cause the value to shift from 11,200 to 16,000 cal.

(From Hadidian and Hoagland (52).)

were reported by Gould and Sizer (56) for the dehydrogenase system of *Escherichia coli*, in which study the same activation energy of 19,400 cal. was obtained for the dehydrogenation of acetate, glycine, glutamate, lactate, succinate, glucose, mannitol, galactose, xylose and sucrose (*cf.* Fig. 4). Results for the aldehyde-purine dehydrogenase system of milk (51) indicated the same activation energy of 16,000 cal. for the dehydrogenation of

acrolein, xanthine, benzaldehyde, piperonal and acetaldehyde. The activation energy for the dehydrogenation of some compounds by the bacterial or milk enzymes, however, was neither 19,400 or 16,000 cal. Hadidian and Hoagland (52, 53), working with a reconstructed enzyme oxidation system of heart, found different activation energies for the dehydrogenation of succinate and *p*-phenylene diamine, although from analogy with inorganic catalysts identical  $\mu$  values would not be expected for such widely different substrates. Butler (59) found nearly the same values for the hydrolysis of two different substrates by chymotrypsin, but diverse values for the hydrolysis of three substrates by trypsin (Table III). Schwartz (60), working with the hydrolysis of the series of triglycerides from tributyrin to tricaprylin by pancreatic lipase, found the same  $\mu$  value of 8700 cal. for all in the presence of excess substrate, although in low substrate concentration the  $\mu$  value increased with dilution. Such spurious effects at sub-maximal substrate concentrations were predicted by Haldane (37), who pointed out that abnormally high activation energies could be accounted for by the influence of temperature upon the accelerated rate of formation of enzyme-substrate complex, in addition to the usual activation energy for the breakdown of the complex. The same activation energies have also been reported for the enzymic hydrogenation of oxalacetate and pyruvate (79) and for the action of tyrosinase on cresol and catechol (57).

Although much more information on the subject is needed before definite conclusions can be drawn, it appears that in many instances the same activation energy characterizes the action of an enzyme on different substrates. Conversely, when the activation energy for the breakdown of two related substrates by an enzyme extract is the same, this identity can be taken as presumptive evidence that the same enzyme is acting as catalyst in both reactions.

##### *5. The Relationship of the Activation Energy of an Enzyme to the Biological System from Which It Was Derived*

The process of extraction of an enzyme from cells might so alter the structure of the enzyme that the activation energy would be modified. Such is not the case for yeast invertase, the properties of which are the same for the intracellular and for the extracted and partially purified enzyme (61). The same activation energy of 11,000 cal. characterizes this enzyme from 0° to 17° whether the enzyme is partially purified or present in dead

cells or in living cells (80, 81).<sup>\*</sup> Similarly, Sizer (47, 48) obtained the same activation energies for crystalline urease, partially purified urease, and for the enzyme still present in the jack bean or soy bean. It thus

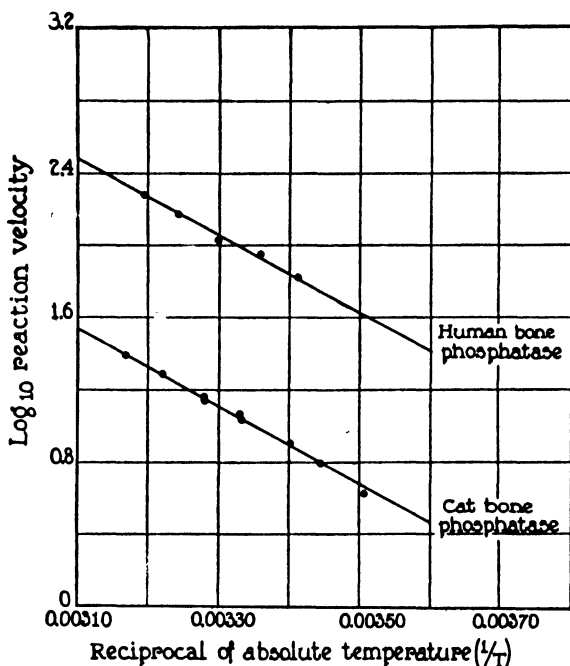


Fig. 7.—From an Arrhenius plot of the data on the hydrolysis of sodium  $\beta$  glycerophosphate by bone phosphatase the activation energy is calculated to be  $9,940 \pm 140$  cal. from  $12-42^\circ$ . Inactivation occurs above  $42^\circ$ . The activation energy is identical for human and cat bone phosphatase. (From Bodansky (42).)

appears that the activation energy is not changed by the extraction of an enzyme from cells or tissues.

Studies on the properties of enzymes indicate that the tissue of derivation does not influence the characteristics of an enzyme. For example, Senter

<sup>\*</sup> Above  $17^\circ$  for intracellular invertase the activation energy decreases abruptly to 8300 cal.

(62) and Nordfeldt (63) reported the same activation energy of 6200 cal. for both blood and fat catalase of beef.

Comparable enzymes from different species are very similar in their chemical and physical properties, but are species-specific proteins which are not identical immunologically according to Northrop (17). Scrutiny of Table IV and Fig. 7 (42) indicates that for the enzymes studied, with the exception of invertase, there is a striking agreement between the activation energy of a particular enzyme obtained from different species of animals and plants. The discrepancy in the case of invertase might be explained by the suggestion that some enzyme in malt other than invertase (*e. g.*, maltase) is responsible for inversion, and thus a different activation energy is obtained. The implications for physiological phenomena of the identity of activation energies of an enzyme from different species are indicated in a subsequent section.

#### 6. *The Effect of Temperature on the Dissociation of the Enzyme-Substrate Complex*

The affinity of enzyme for substrate is measured by the reciprocal of the Michaelis-Menten constant,  $K_m$ , which is considered to be the dissociation constant of the enzyme-substrate complex. The heat of formation of the enzyme-substrate complex may be determined by an analysis of the change in  $K_m$  with temperature, from which may be calculated  $\Delta H$  in the van't Hoff equation. Relatively little work has been done on this interesting problem. Kiese (64) studied the carbonic anhydrase system at four different temperatures and found that  $K_m$  increases with temperature in accordance with the van't Hoff equation where  $\Delta H = -20,000$  cal./mole, and the entropy change is  $-59.8$  cal./degree/mole. The corresponding energy of activation for the carbonic anhydrase system is 8900 cal. For cucumber citric acid dehydrogenase the change in  $K_m$  with temperature corresponds to  $\Delta H = -13,400$  cal./mole (65), for yeast invertase the heat of formation of the enzyme-substrate complex is  $-2000$  cal./mole (66) or 0 cal./mole (67), for the action of emulsin on methyl glucoside  $\Delta H = -7500$  cal., on ethyl glucoside  $\Delta H = -4500$  cal. (16) Haas (82) has reported that the heat of formation of the cytochrome reductase-dihydrotriphosphopyridine nucleotide complex is 2000 cal. The importance of this subject for the interpretation of enzyme reactions certainly justifies further study of the affinity of enzyme for substrate as a function of temperature.

### 7. Entropy Changes Accompanying Temperature Activation of Enzymes

Very little information is available concerning the entropy of activation,  $\Delta S$ , in enzyme-catalyzed reactions, although  $\Delta S$  may be readily calculated from equation (8) which at 0° reduces to

$$k^\circ = 5.7 \times 10^{13} e^{-E/RT} e^{\Delta S/R} \quad (10)$$

Stearn (16) calculated  $\Delta S$  from data in the older literature on enzyme kinetics and obtained values ranging from  $-22$  to  $-78$  with an average\* of  $-59$  cal./degree/mole. Comparable reactions with inorganic catalysts have a much smaller value of  $\Delta S$ . It is suggested that the high loss in entropy of enzyme reactions is due to the loss in "randomness" when the enzyme unites with the substrate to form an intermediary complex. Apparently the acceleration in rate associated with the lower activation energy of the enzyme reaction is partially counteracted by a decrease in entropy (68). A careful study of the problem using trypsin and chymotrypsin has been made recently by Butler (59) (cf. Table III). Unlike the data of Stearn these entropies of activation, with one exception, are in the range ( $-5$  to  $-10$ ) taken as representative of normal reactions (8). The rates of these proteolytic reactions are about what would be expected from the simple collision theory

$$k = Ze^{-E/RT} \quad (6)$$

where  $Z$ , the number of collisions per cc. between the reacting molecules, is given by

$$Z = n_1 n_2 \left( \frac{\sigma_1 + \sigma_2}{2} \right)^2 \left[ 8\pi RT \left( \frac{1}{M_1} + \frac{1}{M_2} \right) \right]^{1/2} \quad (11)$$

where  $n_1 n_2$  are the numbers of reacting molecules per cc.,  $\sigma_1 \sigma_2$  their diameters, and  $M_1 M_2$  their molecular weights. For trypsin the calculated rate (relatively independent of the size of the substrate molecule) corresponds to  $\Delta S = -6$  to  $-8$  cal. Butler points out that, since all of the enzyme surface is probably not active, every collision having sufficient energy will not lead to reaction. He suggests that it is the formation of the enzyme-substrate complex which leads to an enhanced rate, and compensates for the steric factor tending to lower the rate by decreasing the number of collisions which result in activation.

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\* Calculated from his data.

## V. Interpretation of Temperature Effects on Physiological Phenomena in Terms of Activation Energies of Enzyme Systems

At an early date Arrhenius (70) realized that different "life-processes" increased with temperature in accordance with his equation and pointed out the similarity of the values of  $\mu$  for biological processes to those for homogeneous reactions. More recently Crozier (55) and many others (*cf.* 54, 71) have shown that a large number of physiological processes follow this equation over the range of temperature from about 0° to 40°, or until a critical temperature is reached. In the latter case the system is characterized by one  $\mu$  value below and another above this critical temperature. The  $\mu$  value frequently is shifted to a new figure when the physiological environment is changed. The  $\mu$  values are not scattered at random but grouped around certain modes, the most important of these being  $\mu = 16,000, 11,000$  and  $8000$  cal. It is suggested that in the catenary series of catalyzed reactions involved in a physiological process the slowest is the master or pacemaker and determines the  $\mu$  value of the over-all phenomena. A shift in  $\mu$  value is interpreted as indicating that a new step in the series has become the pacemaker. Since in different species the same  $\mu$  values have often been found, Crozier (55) has suggested that reactions catalyzed by certain inorganic catalysts such as the hydroxyl ion ( $\mu = 11,000$ ), the hydrogen ion ( $\mu = 20,000$ ) and iron ( $\mu = 16,000$ ), as well as reactions catalyzed by enzymes (*e. g.*, succinic dehydrogenase,  $\mu = 16,700$ ), may be the pacemakers for many diverse physiological processes in different organisms. The applicability of the Arrhenius equation to physiological phenomena has been questioned by many (72, 73, 74, 76), but see also discussions in (40, 54, 68).

The first demonstration that the  $\mu$  value of a physiological process occurring inside a cell could be explained in terms of enzyme kinetics was made by Sizer (80, 81) with yeast invertase, where it was shown that sucrose hydrolysis inside the cell and in a solution of partially purified invertase was characterized by the same energy of activation. This was followed by work with urease where the activation energy for the hydrolysis of urea was the same inside the jack bean or soy bean as in a solution of crystalline or partially purified urease (47, 48). If enzyme-catalyzed reactions are the pacemaker links in physiological processes, then the activation energies of these enzyme reactions should be alike for the same enzyme from different species, in view of the identity of many  $\mu$  values for physiological processes in different organisms. Such appears to be the case, since the same activation energy for an enzyme from different species



has been demonstrated with a variety of enzymes (*cf.* Table IV). The fact that the same energy of activation characterizes the hydrolysis or oxidation of different substrates by a given enzyme (this does not apply universally, *cf.* previous section) is consistent with the fact that the same  $\mu$  value characterizes different biological phenomena which must involve many different substrates for enzymes.

Attempts of a preliminary nature have been made to apply the information on enzymes to the interpretation of the effects of temperature on biological systems. It is proposed that the frequently recurring  $\mu$  value of physiological systems of 16,700 cal. can be ascribed to the action of succinic dehydrogenase of bacteria (55), the value of 19,400 cal. to the action of the dehydrogenase of *Escherichia coli* (56), and the values of 16,000 and 11,000 cal. to the action of the heart dehydrogenase-cytochrome-cytochrome oxidase system (52). The  $\mu$  value of 11,700 cal. characterizes not only the oxidation of *Lupinus* oil by the fat oxidase of *Lupinus albus*, but the respiration of this plant as well (83). The interpretation of respiratory processes and other physiological phenomena dependent on respiration will be greatly facilitated when each enzymic component of the respiratory chain is isolated and its activation energy determined. Partial and fairly complete reconstruction of this chain and study of temperature effects *in vitro* should eventually lead to an interpretation of the  $\mu$  values for respiration. It does not seem unlikely that eventually the effects of temperature on all rates of physiological phenomena will be interpreted in terms of the activation energies of the enzymic links in the catenary series of events involved in biological processes.

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# X-RAYS AND THE STOICHIOMETRY OF THE PROTEINS

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It is still the considered verdict of most workers in the subject, as it has been since the beginning of the century, that proteins are polypeptide chain systems, alone or in combination with accessory molecules or groupings. This view provides the best and most comprehensive interpretation yet devised. Put more precisely in the light of recent developments, proteins are built in general from *folded* polypeptide chains cross-linked through combinations and interactions between their side chains. The problem of protein structure, therefore, resolves itself in the first place into discovering the nature and numbers of the amino acid residues present, and then, above all, into determining the *configuration* of the chain or chains, and what it is that induces or maintains that configuration. The title of this article suggests that it is concerned with the numerical proportions of the amino acid residues in proteins, and so it is, among other things; but it is not sound structure analysis, nor indeed is it possible, to consider the distribution and properties of these residues apart from the patterns they form, and so in the end we inevitably find ourselves exploring the whole architecture of proteins.

In some ways we know more now about the general plan underlying the architecture of proteins than about their exact chemical constitution. That they are most beautifully planned, and frequently perhaps down to the last atom, is undeniable when we contemplate the elaborate diffraction patterns of feather or porcupine quill for instance, or recall the facts of immunology and admire anew the ultracentrifugal studies of Svedberg and his collaborators, or even when we sift the seemingly discordant chemical data, if we put aside unworthy doubts and do not look for the impossible. The major trouble, though, does for the time being rest with the chemical data, partly on account of inadequate or laborious technique, but also on account of analytical variations well outside the limits of experimental error. The problem has two main aspects, not entirely distinct, but convenient for purposes of discussion: the first concerns the constitution of individual proteins, the second that of groups of proteins of similar molecular plan. It is chiefly with regard to the second that X-ray analysis offers evidence of an *intramolecular* kind, but before leading up to it, it will be best to examine some purely chemical data, mostly, however, on proteins for which there is as yet little intramolecular enlightenment from X-ray sources.

### I. Chemical Evidence on the Proportions of the Amino Acid Residues

The residue weights of the amino acids used in protein structure range from that of glycine (57) to that of tryptophan (186), and their arithmetic mean is about 120. On a rough average then, 100 gm. of protein should contain something of the order of  $100/120 = 0.83$  gram-residues of amino acids, and if the yield of any particular acid is large, it may be possible at once to hazard a sound guess as to what fraction of the total residues arises from that acid. More exactly,

$$100/R = G \text{ and } G/g = f,$$

where  $R$  is the average residue weight (reckoned in accordance with the relative proportions of the different kinds of residues),  $G$  = total gram-residues in 100 gm. of protein,  $g$  = gram-residues of any amino acid in 100 gm. of protein (*i. e.*, the percentage yield of the acid divided by its molecular weight), and  $f$  is the "frequency"\* of the residues in question; by which is meant simply the reciprocals of the fraction of the total residues, and not necessarily that the residues of any one kind are equally spaced along the polypeptide chain or chains.

\* The expression is due to Bergmann and Niemann (1), though with the implication that the residues of any one kind *are* equally spaced.

For example, the leucines yield from hemoglobin has been reported (2, 3) as 27.8 gm. and 29.5 gm., corresponding to 0.21–0.22 gram-residues; and from insulin the leucines yield (4) of 30.0 gm. corresponds to about 0.23 gram-residues; so that in both these cases the suggestion is that one-quarter of the residues are leucine or isoleucine residues. Similarly, from gliadin, 43.0 gm. of glutamic acid (5) corresponds to rather more than 0.29 gram-residues (all in the form of glutamine residues, to judge by the amide nitrogen), with the suggestion that one-third of the residues are glutamine residues: and again, from silk fibroin, the reported yields (6, 7) of 40.5 gm. and 43.8 gm. of glycine, corresponding to 0.54 and 0.58 gram-residues, respectively, indicate that as many as half the total residues in fibroin are glycine residues (since a high glycine content must give a low average residue weight).

With single *low* yields, of course, we can feel no such confidence; nevertheless it has been observed for some time that certain yields, expressed as gram-residues, at least bear a simple numerical relation to one another, and that again, as with the examples just quoted, powers of 2 and 3, or products of such powers, seem to be common. Block (8) pointed out, for instance, that the hemoglobins from the horse, the sheep and the dog all contain iron, histidine, arginine and lysine in the ratio 1:8:3:9, and that in the keratins the ratio of histidine, arginine and lysine is about 1:15:5 (since modified (9) to 1:12:4); while Atkin (10) pointed out that Dakin's results indicate that glycine and hydroxyproline residues are present in gelatin in the ratio of 3:1—and so on. Other examples could be cited, but these will serve to illustrate a growing impression during the past ten years or so.

In 1933 the writer, with H. J. Woods, made the first attempt to link up the ratios of gram-residues with X-ray and physicochemical data on wool keratin (11), and to construct a tentative plan of the distribution of residues along the polypeptide chains. This was followed by the application of similar ideas to the problem of gelatin (12), and on the basis of chemical analyses then available (the proline yield, for instance, was too low) it was concluded that "the residues of the two chief acids, glycine and oxyproline (hydroxyproline), account, respectively, for about one-third and one-ninth of the total number of residues; that is to say, every third residue could be a glycine residue and every ninth an oxyproline residue" (13). The way this conclusion was reached was as follows (13): Since the total nitrogen content of gelatin is 18% and the reported yields of amino acids correspond to a nitrogen content of 15.64%, the difference, 2.36%, must correspond to the acids still unaccounted for, and these are very probably monoamino-

monocarboxylic acids. Hence this 2.36% of nitrogen represents  $2.36/14 = 0.16_6$  gram-residues of undetermined acids. The total reported yields gave a total of 0.87<sub>6</sub> gram-residues in 100 gm. of gelatin, and therefore the true total must be  $0.87_6 + 0.16_6 = 1.04$ , which within the limits of experimental error is three times the number of gram-residues of glycine (0.34) and nine times the number of gram-residues of hydroxyproline (0.11).

Since that time Bergmann and Niemann (1) have proposed two fundamental principles that are generalizations of these early suggestions. They postulate that *all* proteins are built according to the following rules: (1) the numbers of the different kinds of residues and also the total number of

TABLE I

EDESTIN

$$R = 115.7, G = 0.864$$

Amino acid	Yield, %	Gram-residues		Frequency	
		Obs.	Calc.		
Histidine	2.41	0.0156	0.0160	54 (2 <sup>1</sup> 3 <sup>3</sup> )	
Arginine	16.71	0.0961	0.0961	9 (3 <sup>2</sup> )	
Lysine	2.37	0.0162	0.0160	54 (2 <sup>1</sup> 3 <sup>3</sup> )	
Tyrosine (15)	4.34	0.0240	0.0240	36 (2 <sup>2</sup> 3 <sup>2</sup> )	
Tryptophan (15)	1.50	0.0073 <sub>6</sub>	0.0080	108 (2 <sup>3</sup> 3 <sup>3</sup> )	
Methionine (15)	2.35	0.0158	0.0160	54 (2 <sup>1</sup> 3 <sup>3</sup> )	
Cystine/2	1.44	0.0120	0.0120	72 (2 <sup>3</sup> 3 <sup>2</sup> )	
Glutamic acid }	20.7	0.1408	{ 0.0720	12 (2 <sup>2</sup> 3 <sup>1</sup> )	
Glutamine }			{ 0.0720	12 (2 <sup>2</sup> 3 <sup>1</sup> )	
Amide NH <sub>2</sub>	2.15	0.1265	{		
Asparagine }	12.0	0.0902		{ 0.0540	16 (2 <sup>4</sup> )
Aspartic acid }				{ 0.0360	24 (2 <sup>3</sup> 3 <sup>1</sup> )
				L.C.M. = 432 (2 <sup>4</sup> 3 <sup>3</sup> )	

residues in the molecule are always of the form  $2^n 3^m$ , where  $n$  and  $m$  are positive integers, or zero; and (2) the residues of any one kind always occur at a regular periodic interval along the polypeptide chain or chains. We shall proceed to consider some of the chemical evidence for the first of these hypotheses, while postponing judgment on the second till the X-ray evidence has been reviewed.

Quite the most striking evidence for the validity of the  $2^n 3^m$  rule under certain conditions has recently been obtained by Chibnall (14), working with edestin. Chibnall has concentrated on accurate determinations of histidine, arginine, lysine, glutamic acid and aspartic acid, and he estimates the average residue weight from a consideration of the total nitrogen,

the amide nitrogen, and the nitrogen present as histidine, arginine, lysine, hydroxylysine and tryptophan, after the manner of the tentative assessment of gelatin described above (13). In this way he finds for edestin an average residue weight ( $R$ ) of 115.7, corresponding to 0.864 gram-residues per 100 gm. of protein ( $G$ ). Table I is a modified expression of Chibnall's results.

The agreement shown in Table I is so excellent, and what is perhaps even more impressive, there is a *unique* allocation of the amide nitrogen between glutamine and asparagine, that there can be no possible doubt now that for edestin at least, for the amino acids quoted, the  $2^{\circ}3^{\text{m}}$  rule

TABLE II

## EGG ALBUMIN

$$R = 111.5, G = 0.897$$

I—Percentage yield. II—Observed gram-residues in 100 gm. of protein. III—Frequencies referred to 0.897. IV—Minimum molecular weight calculated from I and II. V—Assumed number of residues. VI—Calculated molecular weight of protein.

Amino acid	I	II	III	IV	V	VI
Arginine	5.63	0.03234	27.7	3,092	14	43,280
Histidine	1.45	0.00935	96.0	10,070	4	42,800
Lysine	5.06	0.03463	25.9	2,886	15	43,310
Tyrosine	4.10	0.02264	39.6	4,417	10	44,170
Tryptophan	1.32	0.00647	139	15,460	3	46,400
Cystine (17)	1.79	0.00745	120	13,420	3	40,270
Methionine (17)	5.10	0.0348	26.2	2,925	15	43,900
Glutamic acid	16.1	0.1109	8.1	902	48	43,300
Aspartic acid	8.1	0.0612	14.6	1,638	27	44,100
Amide $\text{NH}_2$	1.23	0.0722	12.4	1,395	31	43,250

holds strictly. The position with regard to the other amino acids must remain open pending further exact analyses, but in the meantime it is clear that there are no fewer than 432 ( $2^43^3$ ) residues in the molecule, and therefore the molecular weight must be  $432 \times 115.7 = 50,000$  approximately, or a multiple thereof. The multiplying factor is presumably 6, since the molecular weight of edestin given by the ultracentrifuge (16) is 310,000.

Chibnall's results for egg albumin (14), on the other hand, by no means give such unequivocal support for the  $2^{\circ}3^{\text{m}}$  rule. His average residue weight for egg albumin comes to 111.5, making a total of 0.897 gram-residues in 100 gm. of protein, and his final results are set out in Table II.

The range of variation among the calculated molecular weights given in



column VI reflects the accuracy of the experimental observations, but most of the determinations are sufficiently near to one another and to the most probable value (about 43,000) given by the ultracentrifuge and osmotic pressure measurements (18) to make it clear that there are undoubted deviations from the  $2^n 3^m$  rule, quite apart from whether the estimated values of  $R$  and  $G$  are correct. The discrepancies are still no proof, however, that the structure of egg albumin does not rest fundamentally on a  $2^n 3^m$  basis, since it has been shown by electrophoresis that many-times recrystallized egg albumin contains two components (19): *both could conform to the  $2^n 3^m$  rule yet contradict it when analyzed together*. We shall meet a similar situation below when we come to consider the case of lactoglobulin; but lactoglobulin is a homogeneous protein, for which there is independent evidence of a number of *intramolecular* components.

The literature of protein analysis, when it is examined constructively and with the eye of stoichiometric faith, is seen to contain many exact agreements, approximate agreements, and seemingly flat contradictions like those just quoted. There would be no point in detailing everything that has been reported, but we wish to present sufficient preliminary chemical evidence for the view that a  $2^n 3^m$  rule must undoubtedly lie at the root of protein structure, though the bald statement that such a rule necessarily governs the analyses of *whole* molecules or structures is an oversimplification, and, indeed, is not to be expected.

**Secretin.**—The analytical data of Ågren (20) on the hormone, secretin, have been re-examined by Niemann (21), who has brought forward reasons for concluding that it is a cyclic polypeptide built from 36 residues. The formula may be written:



where  $L$  = lysyl,  $Ar$  = arginyl,  $P$  = propyl,  $H$  = histidyl,  $Gl$  = glutaminyl,  $As$  = asparaginyl,  $M$  = methionyl, and  $X$  = unknown residues.

**Hemoglobin.**—The analytical data on hemoglobin are still both incomplete and discordant in parts, but for all that it seems clear what stoichiometric scheme is being aimed at, as will appear from Table III, which has been drawn up from a consideration of the more concordant yields, mostly from horse hemoglobin. The total number of gram-residues (0.874) of *protein* (globin) in 100 gm. of hemoglobin has been arrived at by inspection and a process of successive averaging.

The yields shown in Table III come from 100 gm. of hemoglobin, the molecule of which contains four haems with their four iron atoms; and  $100/0.874$  gives us now what may be called a "compensated average residue

weight," from which may be calculated the molecular weight of the complete molecule, without assuming anything about the prosthetic group. Thus:

Let  $M_1$  be the molecular weight of the protein part and  $M_2$  that of the prosthetic part. Then 100 gm. of protein would give  $(M_1 + M_2)G/M_1$  gram-residues, of which the average residue weight is  $100M_1/(M_1 + M_2)G$ .

TABLE III  
HEMOGLOBIN

$R = 110.1$  (for globin),  $G = 0.874$  gram-residues of globin from 100 gm. of hemoglobin

Amino acid	Yield, %	Gram-residues		Proposed frequency
		Obs.	Calc.	
Leucines*	27.8 (22)	0.2122 <sup>+</sup>	0.2185	4 (2 <sup>2</sup> )
	29.5 (23)	0.2251		
Tyrosine	3.15 (24)	0.0174	0.0182	48 (2 <sup>3</sup> 1)
Tryptophan	1.3 (24)	0.0064	0.0061	144 (2 <sup>3</sup> 2)
Proline	(2.3) (22)	(0.0200)		
	2.1 (25)	0.0183	0.0182	48 (2 <sup>3</sup> 1)
Histidine <sup>h</sup>	7.5 (26)	0.0484	0.0486	18 (2 <sup>1</sup> 3 <sup>2</sup> )
Arginine <sup>h</sup>	(3.6) (27)	(0.0207)		
	3.2 (26)	0.0184	0.0182	48 (2 <sup>3</sup> 1)
Lysine <sup>h</sup>	8.1 (26)	0.0555	0.0546	16 (2 <sup>4</sup> )
Glutamic acid <sup>h</sup> }	6.3 (28)	0.0429	{ 0.0243	36 (2 <sup>3</sup> 3)
Glutamine <sup>h</sup> }			{ 0.0182	
Amide N	0.75—	0.054—	{	
	1.07 (29)	0.076		
Asparagine <sup>h</sup> }	8.9 (28)	0.0669	{ 0.0486	18 (2 <sup>1</sup> 3 <sup>2</sup> )
Aspartic acid <sup>h</sup> }			{ 0.0182	
L.C.M. = 144 (2 <sup>3</sup> 3)				

\* Leucine and isoleucine have been grouped together for want of data, though leucine seems to be the constituent generally in excess; but strictly speaking the two should be assessed separately. + These numbers in this and similar tables are of course not significant to four figures, which have been retained for convenience in averaging.

<sup>h</sup> From horse hemoglobin.

Hence if there are  $N$  residues in the molecule, the molecular weight of the protein part is  $100NM_1/(M_1 + M_2)G$ , which is also equal to  $M_1$ . Therefore  $(M_1 + M_2) = 100N/G$ , as before.

The least possible value of  $N$  is 144, but there is so much other evidence that the true value is  $4 \times 144 = 576$ , that we may conclude that the molecular weight of hemoglobin is  $57,600/0.874 = 65,900$ , in quite good agreement with the estimate,  $66,700 \pm 3\%$ , given by the iron content and X-

ray analysis (30). The weight of the globin component in hemoglobin is  $(65,900 - 2472) = 63,400$ , approximately, and its average residue weight is  $63,400/576 = 110.1$ .

**Yellow Enzyme** (31).—The yellow enzyme also presents a plausible case of the  $2^{\circ} 3^{\text{m}}$  rule, as will be seen from Table IV.

An interesting point is that both aspartic and glutamic acids, if the above suggested allocation of the amide nitrogen is correct, are represented only by their amides. There is some inconsistency with regard to the molecular weight, the values 78,000 and 82,000 being reported as given by the

TABLE IV  
YELLOW ENZYME

$$R = 118, G = 0.847$$

Amino acid	Yield, %	Gram-residues		Proposed frequency
		Obs.	Calc.	
Alanine	8.2 (32)	0.0921	0.0941	9 (3 <sup>2</sup> )
Serine	1.7 (32)	0.0162	0.0176	?48 (2 <sup>4</sup> 3 <sup>1</sup> )
Phenylalanine	5.75 (33)	0.0349	0.0353	24 (2 <sup>3</sup> 3 <sup>1</sup> )
Tyrosine	7.75 (33)	0.0428	0.0471	18 (2 <sup>1</sup> 3 <sup>2</sup> )
Tryptophan	4.9 (33)	0.0240	0.0235	36 (2 <sup>3</sup> 3 <sup>2</sup> )
Cystine/2	0.34 (33)	0.0028	0.0029	?288 (2 <sup>8</sup> 3 <sup>2</sup> )
Aspartic acid	2.0 (33)	0.0150	...	...
			0.0235	?36 (2 <sup>3</sup> 3 <sup>2</sup> )
	3.0 (32)	0.0226	...	...
Glutamic acid	7.1 (33)	0.0483	0.0529	?16 (2 <sup>4</sup> )
Histidine	2.75 (33)	0.0177	0.0176	48 (2 <sup>4</sup> 3 <sup>1</sup> )
Arginine	8.25 (33)	0.0474	0.0471	18 (2 <sup>1</sup> 3 <sup>2</sup> )
Lysine	13.7 (33)	0.0938	0.0941	9 (3 <sup>2</sup> )
Amide N	1.07 (33)	0.0764 =	0.0235 +	?36 (2 <sup>3</sup> 3 <sup>2</sup> )
			0.0529	?16 (2 <sup>4</sup> )

ultracentrifuge (16, 34, 35); but from Table IV the minimum number of residues is 144 (2<sup>4</sup>3<sup>2</sup>), or possibly 288 (2<sup>8</sup>3<sup>2</sup>), corresponding to a minimum molecular weight of 34,000 which is supported by estimates of the flavin content (lactoflavin-5-phosphate). Kuhn and Desnuelle (33) found  $8.25 \times 10^{-5}$  gram-molecules of prosthetic group in 5.8 gm. of the enzyme and therefore a molecular weight for the latter of about 70,000, while Theorell (36) also found 70,000–75,000 from a photoelectric determination.

**Caseinogen.**—It is possible to make out a reasonable case for a value of  $G$  equal to about 0.805 and a minimum molecular weight of about 53,700, corresponding to 432 (2<sup>4</sup>3<sup>3</sup>) residues (*cf.* edestin). The yields of

histidine (37, 38), arginine (37, 38, 27) and threonine (39), for instance, leave little doubt that their respective frequencies are 72, 36 and 27.

**Salmine.**—This protamine consists mostly of arginine residues, together with much smaller amounts of valine, serine and proline (40). The average residue weight computed directly from the yields of these four acids comes out to be about 138, corresponding to  $G = 0.726$ . The reported arginine yield is 87.4%, or 0.502 gram-residues, from which it follows that the arginine residues almost certainly account for an exact two-thirds of the whole. Two possible formulas for the molecule are:



or



where  $Ar$  = arginyl,  $Pr$  = prolyl,  $Se$  = seryl,  $V$  = valyl, and  $X$  = an unknown residue.

It must be emphasized that there is no intention in these introductory illustrations of the trend of protein stoichiometry to insist on the detailed validity of every interpretation here put forward. Chibnall's results with edestin are irresistible, the scheme suggested for hemoglobin is probably sound in the main, and that outlined for the yellow enzyme is plausible, for instance; but there is no lack of examples which carry little conviction yet, either one way or the other. No doubt much of the trouble with these is due to imperfect or incomplete analyses, or to the use of preparations that are not protein individuals; but beyond all these things there is still to be found a residuum of apparently erratic stoichiometry, and there is still the problem of the fibrous proteins, where so few structural types are adapted to so wide a range of chemical constitution. We shall continue now, in fact, our consideration of the  $2^n3^m$  rule in the field of the fibers, because, as already mentioned, they alone so far provide X-ray data at all interpretable in the light of a dawning stoichiometry. Afterward we shall return to the nonfibrous proteins.

To summarize, then, the lesson to be learned from data of the kind presented above, we may say that there is now enough chemical evidence to warrant the belief that the structural plan used in building the constituent parts of protein molecules, and sometimes, or maybe often, in building whole protein molecules, involves proportions of amino acid residues in the ratios of values of  $2^n3^m$ , where  $n$  and  $m$  are positive integers, or zero.

## II. The X-Ray Classification of the Fibrous Proteins (41)

The fibrous proteins give rise to only a few different kinds of X-ray dif-

fraction pattern. All sorts of fibers have been examined, but fundamentally they seem to fall into no more than two main structural groups, the keratin-myosin group and the collagen group. The principal subsection of the keratin-myosin group includes the fibrous proteins of the epidermis of mammals (42), amphibians and certain fishes (43), and the fibrous structures, such as hair, horn, nails, etc., that arise from the mammalian epidermis. These are all so-called keratinous products, and the prototype X-ray photograph is that of normal, unstretched mammalian hair. On stretching, keratin fibers give rise to another type of diffraction pattern, but this reverts to the normal when the fibers are allowed to contract again. The phenomenon is one of reversible, long-range elasticity inherent in the molecules themselves. In the unstretched state keratin consists of regularly folded polypeptide chains running in the direction of the fiber axis, but stretching the fiber pulls these chains out almost straight, though they return to their folded configuration when released in the presence of water or other polar molecules. We have called the normal unstretched form  $\alpha$ -keratin, and the extended form  $\beta$ -keratin (44).

Myosin, the chief protein component of muscle tissue and the seat of its elastic properties, gives also an X-ray photograph of the  $\alpha$ -keratin type. It gives, too, a  $\beta$ -photograph when stretched—in fact, put briefly, we may say that all the principal X-ray photographs and elastic properties of keratin find their counterparts in myosin, and there is no doubt at all now that the two proteins belong to the same configurational group (45).

The  $\beta$ -subgroup of the keratin-myosin group includes also fibroin (46) and fibrin (47), and structures such as feathers (48), tortoise shell, scales and the like, that spring from the epidermis of birds and reptiles (49). The X-ray photograph of silk fibroin is readily distinguishable from that of  $\beta$ -keratin but that of fibrin rather resembles it, while the typical feather keratin pattern is superficially different from either: all three, however, are to be explained on the basis of polypeptide chains that are stereochemically fully extended, or approximately so. The feather keratin structure is the most contracted of the three, the chains being some 7% shorter than the observed maximum, which is shown by fibroin.

Artificial fibrous proteins (50, 51), made, for example, by spinning the viscous solutions obtained by the denaturing action of strong urea on the seed globulins, give when stretched X-ray photographs that are again of the  $\beta$ -type. Here the polypeptide chains are first unfolded from their native configuration and then drawn parallel.

The collagen fibers constitute the other great group of natural fibrous proteins—and by collagen fibers is meant all such things as the white con-

nective tissue fibers, tendons, cartilage, the scales and fins of fishes, the ichthyocol of swim bladders, jellyfish, the derived protein gelatin, and many other structures (52), all of which give a diffraction pattern of a type quite different from that of any member of the keratin-myosin group. The polypeptide chains are held inextensibly in a configuration that is about 20% short of the maximum length, a stereochemical peculiarity that we shall return to below, after discussing the nature of the  $\alpha$ -fold in the keratin-myosin group.

Both the keratin-myosin group and the collagen group, though each is characterized by a constant molecular plan, show wide variations of chemical constitution, and this fact is the crux of their stoichiometry and the subject of much of the rest of this article. As we pass down the keratin-myosin group from keratin through the epidermal proteins to myosin there is a continuous gradation in chemical constitution that is beyond question—yet the molecular configuration and elastic properties remain substantially the same: and similarly for the members of the collagen group.

We are now in a position to discuss the chief types of protein fibers in turn in relation to both the X-ray and chemical findings. When this has been done, it will be convenient to conclude with some tentative suggestions—for that is all that is possible at the moment—in the field of the non-fibrous proteins.

### III. Silk Fibroin

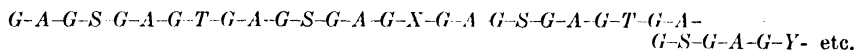
The reported amino acid yields from silk fibroin appear to be nearly complete: they add up to a maximum of about 1.16 gram-residues per 100 gm. of protein, which is approximately twice, four times, eight times and sixteen times the number of glycine, alanine, serine and tyrosine residues, respectively. The best value of  $G$  seems to be about 1.17 gram-residues, corresponding to an average residue weight of 85.5. The relevant analytical data are set out in Table V, from which it will be seen that there is quite strong evidence for the proportions of glycine, alanine, serine and tyrosine just mentioned. There remains only one-sixteenth to be apportioned among the other residues: the yields of these are too small, however, to be quite sure what their corresponding fractions are; they may, in fact, be partly extraneous to the main stoichiometric scheme.

It is tempting to suppose that this scheme is based on the series:

$$- \quad \frac{1}{2} + \frac{1}{4} + \frac{1}{8} + \frac{1}{16} + \frac{1}{32} + \frac{1}{64} + \text{etc.},$$

the sum of which to infinity is unity; that is to say, that the polypeptide

chains are essentially of the form:



where *G* = glycyl, *A* = alanyl, *S* = seryl, *T* = tyrosyl, and *X*, *Y*, etc., = residues not yet defined.

Presumably the data in Table V refer all to the commoner kind of silk, *Bombyx mori*. There is a fair amount of X-ray results available for this

TABLE V

## SILK FIBROIN

$$R = 85.5, G = 1.17$$

Amino acid	Yield, %	Gram-residues		Proposed frequency
		Obs.	Calc.	
Glycine	43.8 (7)	0.5840	0.5850	2 (2 <sup>1</sup> )
Alanine	25.0 (6)	...	...	...
	26.4 (7)	0.2966	0.2925	4 (2 <sup>2</sup> )
Serine	13.6 (53)	...	...	...
	15.5 (54)	0.1475	0.1463	8 (2 <sup>3</sup> )
Tyrosine	13.2 (7)	0.0729	0.0731	16 (2 <sup>4</sup> )
Threonine	1.4 (53)	0.0118	...	...
	0.8 (54)	0.0066	...	...
Leucines	2.5 (6)	0.0191	0.0183	?64 (2 <sup>6</sup> )
Phenylalanine	1.5 (6)	0.0091	...	...
Proline	1.0 (6)	0.0087	...	...
Histidine	0.07 (55)	0.0005	...	...
Arginine	0.7 (55)	0.0040	...	...
	0.76 (27)	0.0044	...	...
	0.95 (7)	0.0055	...	...
Lysine	0.25 (55)	0.0017	...	...
Glutamic acid	0 (6)	0	...	...

fiber (46), but unfortunately they are hardly decisive on a number of points. The intramolecular pattern repeats along the fiber axis at a distance of  $2 \times 3\frac{1}{2}$  A.,\* and it was Meyer and Mark who first interpreted this as meaning that fibroin is built from fully extended chains of residue length equal to  $3\frac{1}{2}$  A., a view that is now accepted. Kratky and Kuriyama have worked out various possible unit cells and drawn the general conclusion that on the average the chains are no less than 4.5 A. and no more than 6.1 A. apart. Their favored unit cell has the dimensions: (56)

$$a = 9.68 \text{ A.}, b = 7.00 \text{ A.}, c = 8.80 \text{ A.}, \beta = 75^\circ 50',$$

\* In this paper the abbreviation A. for the Ångstrom Unit has been used.

and the density of the fiber is given as 1.33 to 1.46 gm./cc. Meyer and Mark, on the basis of analyses then available, assumed that glycine and alanine residues are present in equal proportions and that the cell contains four of each; and granted these things the argument is reasonable, for the volume of the cell is 578.3 A.<sup>3</sup>, and therefore the average residue weight must lie between

$$\frac{578.3 \times 1.33}{8 \times 1.65} \text{ and } \frac{578.3 \times 1.46}{8 \times 1.65};$$

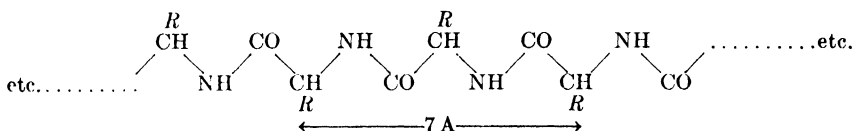
that is, between 58.3 and 64. For equal proportions of glycine and alanine the average residue weight would be 64.

In the whole fiber, however, there are not equal proportions of glycine and alanine and the average residue weight is about 85.5, which leads to an average residue volume between

$$\frac{85.5 \times 1.65}{1.33} \text{ A.}^3 \text{ and } \frac{85.5 \times 1.65}{1.46} \text{ A.}^3;$$

that is, between 106.1 A.<sup>3</sup> and 96.6 A.<sup>3</sup>; and the volume of the chosen cell is about 5½ and 6 times these numbers, respectively. Unless we are prepared to say that the more crystalline part of the fiber is built solely from glycine and alanine residues in equal proportions and to relegate the rest of the glycine and all the other residues to an amorphous, intercrystalline phase, the conclusion seems to be that the chosen unit cell is wrong, and that its volume is perhaps either ¾ or 1½ times the volume of the true cell.

Obviously, silk fibroin needs to be re-attacked by X-rays with more up-to-date technique—and quite apart from the apparent discrepancy in the matter of the unit cell, there is the important point that no period longer than  $2 \times 3\frac{1}{2}$  A. has yet been reported for the pattern along the fiber axis, whereas, if the residues were distributed in regular periodic fashion along the polypeptide chains as set out above, we might expect to detect a pattern repeating at perhaps up to  $16 \times 3\frac{1}{2}$  A. Again we could explain this absence of a long period by postulating the same crystalline and intercrystalline phases of special constitution, but it seems more likely that the residues do not follow one another always or exactly in strict periods, so that in the end the resultant observed period expresses no more than that the residues point alternately to one side and the other of the chain, thus:





If this is the correct interpretation—and to summarize, we have to state that the X-ray evidence to date does not support the view that the residues in silk fibroin follow one another always or exactly in the periods suggested by their relative proportions—we are left with the interesting corollary that in the synthesis of a protein the amino acids may be supplied in definite proportions without the guarantee that they will be put together with equivalent regularity: in other words, the over-all proportions of residues could be decided by the interrelations of a set of supply reactions, while

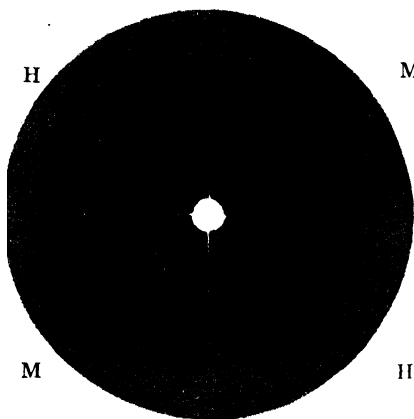


Fig. 1a.—X-ray sector comparison photograph of  $\alpha$ -horn and  $\alpha$ -myosin (M, myosin; H, horn).

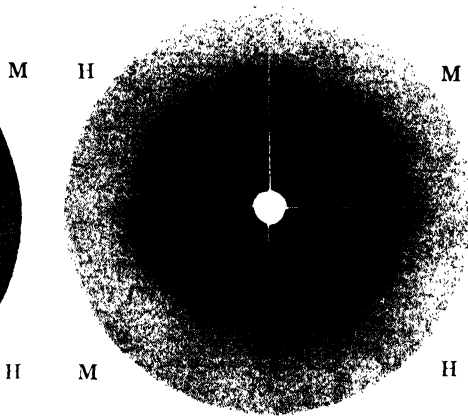


Fig. 1b.—X-ray sector comparison photograph of  $\beta$ -horn and  $\beta$ -myosin (M, myosin; H, horn).

Cu  $K\alpha$  rays.

Each of these figures corresponds to two photographs taken on one and the same film, diagonally opposite quadrants being part of the same photograph. Each meridional or equatorial reflection is thus divided into two halves, one half arising from keratin and the other from myosin.

their ultimate pattern and configuration, and therefore the specific properties of the protein, might be determined by a series of independent operations. In fibroin these latter operations would be simply irregular condensation.

#### IV. The Keratin-Myosin Group (57, 58)

It will be evident from Figures 1 (a) and 1 (b) how very much alike the X-ray diffraction patterns of keratin and myosin are: they are not identical

in fine detail, of course—it would be unjustifiable to expect that—but they have so much in common as to leave no doubt, especially when we remem-

TABLE VI  
WOOL KERATIN

$$R = 118, G = 0.85$$

I—Amino acid. II—Reported yields from 100 gm. of wool. III—Yield chosen as probably most nearly correct. IV—Gram-residues in 100 gm. of wool.

I	II	III	IV
Glycine	0.58 (59), 6.5 (61)	6.5	0.0870
Alanine	4.13 (59)	4.13	0.0464
Valine	2.8 (59), 4.8 (62) <sup>m</sup>	4.8	0.0410
Leucines	11.5 (59), 11.3 (62) <sup>bm</sup>	11.3	0.0863
Phenylalanine	4.0 (61), 3.75 (62) <sup>m</sup>	3.75	0.0227
Proline	4.4 (59), 6.8 (62) <sup>m</sup>	6.8	0.0591
Methionine	0.44–0.66 (63), 0.7 (62) <sup>m</sup>	0.7	0.0047
Cystine/2	7.3 (59), 13.1 (60, 61), 11.9 (66) <sup>f</sup>	11.9	0.0989
Serine	0.1 (59), 10.3 (62) <sup>cm</sup>	10.3	0.0981
Threonine	6.4 (62) <sup>cm</sup>	6.4	0.0538
Tyrosine	2.9 (59), 4.8 (60), 4.5 (61)	4.65	0.0257
Aspartic acid	2.3 (59), 5.84 (66), 7.3 (64) <sup>c</sup>	6.57	0.0494
Glutamic acid	12.9 (59), 15.3 (64) <sup>c</sup>	14.1	0.0959
Arginine	10.2 (60), 8.7 (61), 10.4 (65)	10.3	0.0592
Lysine	2.8 (60), 2.5 (61)	2.65	0.0182
Histidine	6.9 (60) <sup>a</sup> , 0.7 (61)	0.7	0.0045
Tryptophan	1.8 (60), 0.7 (61)	1.8	0.0088
Total gram-residues in 100 gm. of wool			0.8597*
Amide N	1.2 (60), 1.37 (62), <sup>cm</sup> 1.37 (64), <sup>c</sup> 1.14 (67), <sup>cmd</sup> 1.12 (67) <sup>ce</sup>	1.13	0.081

<sup>a</sup> Without doubt excessive. <sup>b</sup> Martin and Syngé's work suggests a leucine : isoleucine ratio of about 4 : 1. <sup>c</sup> Cotswold wool. <sup>m</sup> Merino wool. <sup>d</sup> 1.37 value corrected for decomposition of serine. <sup>e</sup> Mild hydrolysis. <sup>f</sup> Estimated as total —S—S— and —SH.

\* Not, of course, significant to four figures. These have been retained throughout so as to average out in the addition.

ber the similarity between the elastic properties of the two fibers, that they arise from a common molecular plan.

Tables VI and VII (adapted from Tables II and III in *J. Chem. Soc.* (58)\*) collect together all the available analytical data for wool keratin

\* Unfortunately there is an error in Table III of reference (58), in that the number of gram-residues of glutamic acid plus amide is given as 0.1469, whereas it should be 0.1504; and the total yield comes not to 0.7825, but to 0.7860.

and rabbit myosin. At first sight it might appear rather hopeless, particularly in view of the element of incompleteness and uncertainty, to try

TABLE VII

## RABBIT MYOSIN

$$R = 116, G = 0.86$$

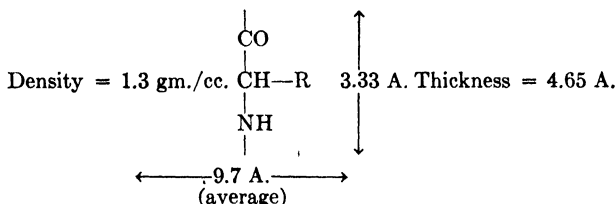
I—Amino acid. II—Most recently reported, and probably best, yields from 100 gm. of myosin. III—Gram-residues in 100 gm. of myosin.

I	II	III
Cystine/2 + cysteine	1.39 (69)	0.0116
Methionine	3.4 (68)	0.0228
Serine	3.57 (62)	0.0340
Threonine	3.81 (62)	0.0320
Tyrosine	3.4 (68)	0.0188
Aspartic acid	8.9 (70)	0.0669
Glutamic acid	22.1 (70)	0.1504
Arginine	7.0 (70)	0.0402
Lysine	10.3 (71)	0.0705
Histidine	1.7 (70)	0.0110
Tryptophan	0.82 (68)	0.0040
.....	.....	.....
Glycine	1.9 (70)	0.0253
Alanine	5.1 (71)	0.0573
Valine	...	...
Leucines	...	...
Phenylalanine	...	...
Proline	...	0.2412 <sup>b</sup>
Total		0.7860
Amide N	1.19 <sub>3</sub> (71), <sup>a</sup> 1.19 <sub>6</sub> (69) <sup>a</sup>	0.0852

The yields quoted in the upper half of the table are more reliable than those quoted in the lower half.

<sup>a</sup> Mild hydrolysis. <sup>b</sup> Sharp's yields of these four acids give together 0.1506 gram-residues, but Martin and Synge (72), using their new chromatographic method, have estimated for me that these four together with methionine account for probably some 22% of the total nitrogen. From Sharp's value of the total N (16.8%), and allowing for the methionine yield quoted above, this means that valine, leucines, phenylalanine and proline give altogether 0.2412 gram-residues. Sharp recognizes, however, that most of his shortage lies with the monoamino acids, and the 9% still wanting may reasonably be ascribed chiefly to the glycine and alanine determinations.

to co-ordinate them with the X-ray data on keratin and myosin, but actually the latter give us at once the average residue weight, as follows:

**$\beta$ -Keratin**

Average residue mass =  $3.33 \times 9.7 \times 4.65 \times 1.3 \times 10^{-24}$  gm.

One-sixteenth of the mass of an oxygen atom =  $1.65 \times 10^{-24}$  gm.

Therefore average residue weight =  $\frac{3.33 \times 9.7 \times 4.65 \times 1.3}{1.65} = 118$  (approx.); and

number of gram-residues in 100 gm. of keratin = 0.85.

Similarly for

 **$\beta$ -Myosin**

Average residue weight =  $\frac{3.3 \times 9.8 \times 4.65 \times 1.275}{1.65} = 116$  (approx.), and number of gram-residues in 100 gm. of myosin = 0.86.

From these calculations we see that, within certain limitations (58), the amino acid analyses of wool keratin are now reasonably sound and complete—the yields chosen as most plausible add up, as a matter of fact, to rather more than the predicted. For myosin the total reported yields still amount to only about 91% of the possible maximum. In both cases we shall base our subsequent calculations on the X-ray value of the average residue weight.

The molecular plan common to the keratin-myosin group will have only an approximately constant weight because of variations in chemical constitution, but it should be founded on always the same number of amino acid residues. We have no decisive argument at the moment as to what this number is, but the indications point to 576 ( $2^9 3^2$ ). The general chemical evidence hinting at some value of  $2^n 3^m$  has already been reviewed above—and in this connection mention should be made also of the recent stimulating findings of Pacsu (73) on the poly-condensation of certain peptide esters into chains of  $3 \times 2^n$  residues—but there are the following special reasons for choosing 576:

1. Myosin incorporates as part of its more permanent make-up a small proportion of phosphorus. Bate Smith and Davis (74) have reported percentages ranging from 0.04 to 0.06, and Bailey's (75) determinations include 0.043–0.050, 0.054, 0.055, 0.055, 0.048 and 0.067. The percentage corresponding to 576 residues of average weight 116 is 0.0463.

2. Column I of Table VIII (from Table IV of *J. Chem. Soc.* (58)) shows the frequencies of the various amino acid residues in wool keratin corre-

sponding to the chosen experimental yields listed in Table VI. They are remarkably close to the powers of 2 and 3 shown in column II, which give residue numbers totaling exactly 576 (column IV). (The residue numbers shown in column III are derived from the experimental frequencies in column I, and so add up to rather more than 576 because the total of the chosen experimental yields is a little too high—see above.)

TABLE VIII  
WOOL KERATIN

I—Experimental frequencies. II—Possible “ideal” frequencies corresponding to I. III—Experimentally determined approximate numbers of residues referred to a total of 576. IV—Numbers of residues, totaling exactly 576, corresponding to the “ideal” frequencies given in I.

Amino acid	I	II	III	IV
Glycine	9.8	9	59	64
Alanine	18.3	18	31	32
Valine	20.7	24	28	24
Leucines	9.9	9	58	64
Phenylalanine	37.4	36	15	16
Proline	14.4	16	40	36
Methionine	181	192	3	3
Cystine/2	8.6	9	67	64
Serine	8.7	9	66	64
Threonine	15.8	16	36	36
Tyrosine	33.1	36	17	16
Tryptophan	96.3	96	6	6
Aspartic acid	17.2	16	34	36
Glutamic acid	8.9	9	65	64
Arginine	14.4	16	40	36
Lysine	46.7	48	12	12
Histidine	188	192	3	3
Total	...	...	580	576
Amides	10.5	...	55	...

Provisionally then, we may suppose that the smallest “pattern weight” in wool keratin is about  $576 \times 118 = 68,000$ , and in rabbit myosin  $576 \times 116 = 66,800$ .

We are not yet in a position to draw up for myosin as complete a table as Table VIII, but Table IX (amplified from reference (58) so as to include a proposed “ideal” distribution of the amides) shows the experimental numbers of residues for the more reliable amino acids in myosin, side by side with corresponding keratin numbers abstracted from Table VIII in order to

facilitate comparison. In what follows, partly to disarm criticism on the score of making free with debatable Bergmann-Niemann numbers and partly because the argument we shall develop makes it unnecessary to assume that such numbers occur infallibly, *we shall use only experimental numbers of residues*. Tables VIII and IX show at a glance the sort of stoichiometric problem with which we are confronted. We have to corre-

TABLE IX

COMPARATIVE CHEMICAL CONSTITUTIONS OF WOOL KERATIN AND RABBIT MYOSIN FOR THE MORE RELIABLE AMINO ACIDS

Amino acid	Observed approximate numbers of residues in 576	
	Wool keratin	Rabbit myosin
Cystine/2 + cysteine	67	8
Methionine	3	15
Serine	66	23
Threonine	36	21
Tyrosine	17	13
Tryptophan	6	3
Aspartic acid + amide	34	45
Glutamic acid + amide	65	101
Arginine	40	27
Lysine	12	47
Histidine	3	7
Amides	55	57

Proposed "ideal" distribution of amides

	Prop.	Obs.	Prop.	Obs.
Aspartic acid	12	34	24	45
Asparagine	24 } 36		24 } 48	
	56		56	
Glutamine	32	65	32	57
Glutamic acid	64 } 64		96 } 96	
	32		64	101

late this wide range of chemical constitution with a common molecular plan and similar long-range elastic properties.

The thesis we wish to develop is that within the framework of the common molecular pattern there is scope for variability among the residues, or groups of residues, analogous to what has long been known to hold in the silicates, alloys and mixed crystals in general. With the data set out in Table IX, however, no correlation based on individual amino acids seems

possible: something broader is required. To explain the constitution and properties of the keratin-myosin group we need to invoke some structural property of supreme generality.

The new model (57) for  $\alpha$ -keratin and  $\alpha$ -myosin certainly has this property of generality. It is based on the polypeptide "grid" and the close-packing of side chains, and it was derived from crystallographic, elasticity and density considerations without reference to any particular side-chain distribution. A satisfactory atomic model (57) has been constructed to scale (see Figures 2 (a) and 2 (b)), but the diagrammatic representation in Figure 3 will best serve our purpose here. The important point to notice is that the side chains are packed together in triads that occur first on one

TABLE X

SUMMATION OF POLAR SIDE CHAINS IN KERATIN AND MYOSIN (EXPERIMENTAL VALUES)

Amino acid	Nos. of residues	
	Keratin	Myosin
Arginine	40	27
Lysine	12	47
Histidine	3	7
Aspartic acid + amide	34	45
Glutamic acid + amide	65	101
Serine	66	23
Threonine	36	21
Tyrosine	17	13
Total	273	284

In myosin there are also on the average about 2 cysteine residues (77).

side of the main chain and then on the other; but we shall now postulate further that the great common theme running through the whole of the keratin-myosin group is simply this, that in the main, *individual side chains are alternately polar and nonpolar*.

This is equivalent to saying that the stoichiometry of the keratin-myosin group is a broad stoichiometry of *types* of amino acids—as indeed it must be in order to generate similar properties from differences in chemical detail—types that are allotted certain *sites* in the molecular plan; and the rule just proposed for the relative distribution of the two broadest possible types offers to co-ordinate the principal features of the diffraction patterns, the long-range elasticity, and the chemical constitution all in a single stroke. The paramount considerations on which it rests are as follows: (a) The triads are formed from individual side chains that lie alternately on

one side and the other of the main chain. (b) Gordon, Martin and Synge (76) find that in the dipeptides formed on incomplete hydrolysis of wool the basic amino acids, at least, are linked with all sorts of nonpolar amino acids—in fact, there seems to be no really outstanding difference between the distribution of acids in the dipeptides and that in the original polypeptide. (c) Polar side chains will tend to aggregate with polar side chains, and nonpolar with nonpolar. If individual side chains are alternately polar and

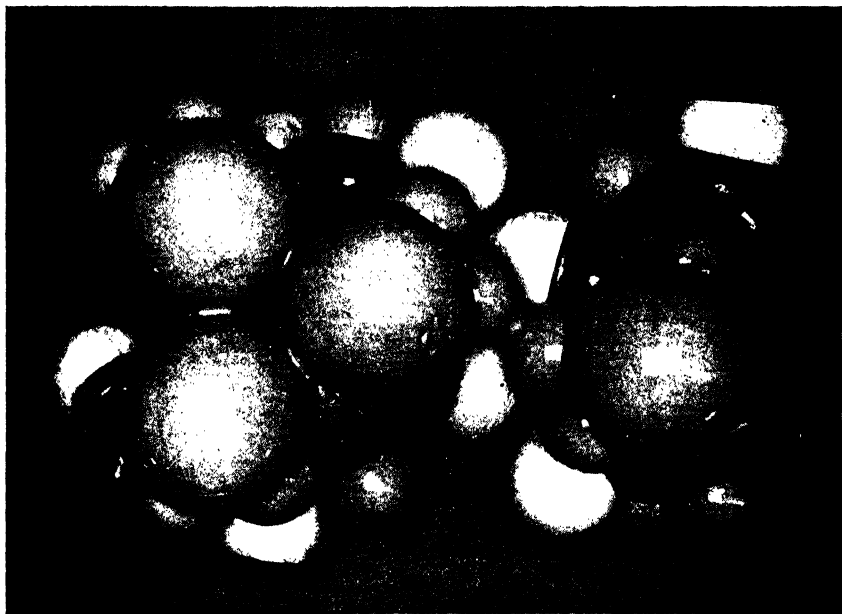


Fig. 2a.—Atomic model, to scale, illustrating the packing of side chains in  $\alpha$ -keratin and  $\alpha$ -myosin. The large spheres represent the second  $\text{CH}_2$ -group counting from the main chain. The region of the structure covered corresponds to the dotted rectangle in Figure 3.

nonpolar, we see that not only do we satisfy condition (b), and, because of (a), bring about (c) automatically, but also we endow the structure with potential long-range elasticity!

A necessary condition, of course, for the regular alternation of polar and nonpolar side chains is that they shall be equal in number. Table X shows that this condition does hold approximately for both keratin and myosin—fortunately we can draw up such a table without worrying about



inadequate data on the nonpolar side chains, since it happens that the estimates of all the polar side chains are sufficiently good. We have no right to insist on exact agreement, partly because of analytical errors that have doubtless not yet been eliminated from Table X, but chiefly because what we have just postulated about the sequence of side chains, though fundamental, may not be the whole story: it may be that the plan is adhered to in general, but certain divergences are permissible in detail, or are essential to serve ends that we do not yet appreciate.

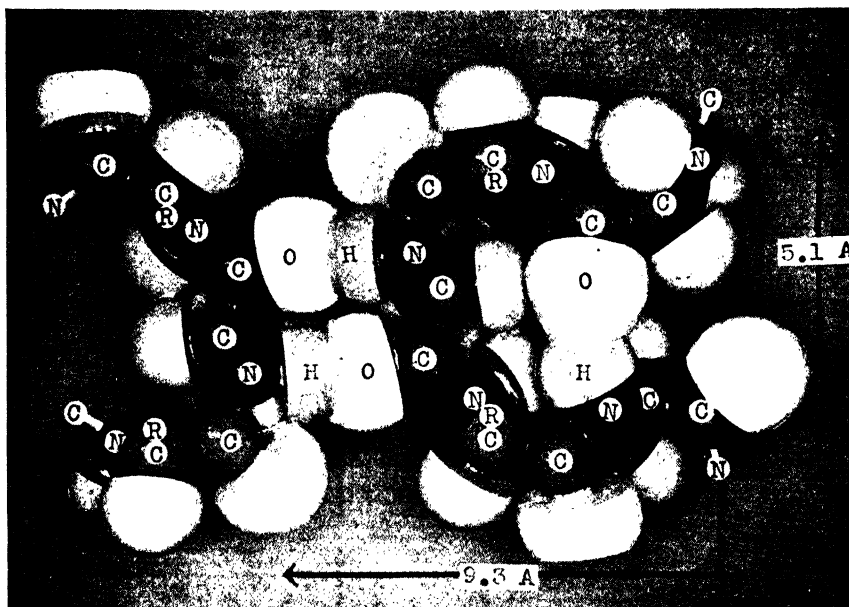


Fig. 2b.—The other face of the same model, with the side chains removed so as to reveal the form of the main chain. (From Astbury and Bell, *Nature*, **147**, 696 (1941).)

As for the explanation the theory offers of the long-range elasticity of the keratin-myosin group, it is easy to raise points of detail to which there are no immediate answers, but nevertheless the new outlook appears more promising in scope than anything that has gone before. It must be repeated that we have to explain similar long-range elastic properties and the same  $\alpha,\beta$ -transformation in the face of startling variations in chemical constitution, and we are suggesting quite broadly that the phenomenon is to be traced throughout the group to a common mode of side-chain aggregation

involving the formation of polar triads on one side of the folded main chain and nonpolar triads on the other. In this scheme there is room for an eventual interpretation of the contraction of muscle in terms of increased

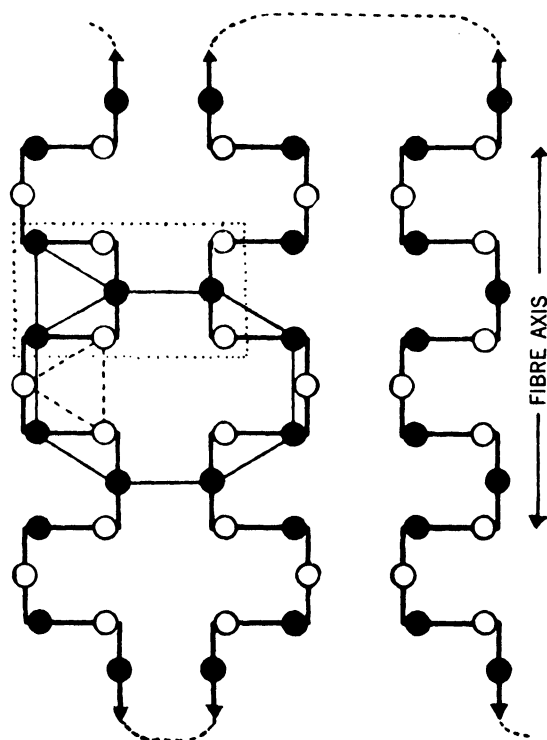


Fig. 3.—Diagrammatic representation of the packing of side chains in the  $\alpha$ -fold of the keratin-myosin group; — represents the direction of the main chains; ● represents a side chain pointing up from the plane of the diagram; ○ represents a side chain pointing down from the plane of the diagram. (From Astbury and Bell, *Nature*, **147**, 696 (1941).)

facilities for aggregation by comparatively simple reversible changes in the relative distributions of polar and nonpolar side chains, and, above all, there is a prospective harmonizing of the concepts that myosin is both the working elastic mechanism in muscle and also a principal enzyme

(adenosine triphosphatase (78)) in the elastic cycle. By the incorporation of specific reactive groupings among the polar side chains the characteristic elastic features of the group would rest unimpaired, but the versatility or sensitivity of the system might very well be increased in relation to a particular chemical environment.

With regard to the constitution of the individual polar triads, on which so largely depend the power of aggregation and the strength of inter-chain linkage, there must also be some rational scheme of combination or interaction between the polar side chains of neighboring main chains in the polypeptide grid; and for such a scheme there are available side chains

TABLE XI  
COMPARATIVE DISTRIBUTION OF POLAR SIDE CHAINS IN KERATIN AND MYOSIN

	Keratin		Myosin	
	Analysis	Titration	Analysis	Titration
Basic	55	51-55 (79) <sup>a</sup>	81	90 (80), 98 (81), 100 (82)
Acidic	44	...	89	87 (82)
Amide	55	...	57	...
Hydroxyl	119	...	57	...

Suggested approximate scheme of cross-linkage between opposed triads in keratin:

1. Acid-base.
2. Amide-hydroxyl (with some replacement by acid-base).
3. Hydroxyl-hydroxyl (with some deficiency probably made up by cystine linkages).

Suggested approximate scheme of cross-linkage between opposed triads in myosin:

1. Acid-base (with some replacement by amide-hydroxyl).
2. Amide-hydroxyl.
3. Acid-base (with some replacement by amide-hydroxyl).

<sup>a</sup> A variety of wools and human hair. The best titration value for Cotswold wool (83) is probably 79.6 cc. of *N* HCl per 100 gm. of wool, corresponding to approximately 54 basic side chains.

whose end groups are acidic, basic, hydroxyl or amides. Besides saltlike linkages between the bare acidic and basic side chains, there can be variety of polar attractions (or hydrogen bridges), and principally we may expect the latter to lie between amide and hydroxyl end groups and between hydroxyl and hydroxyl end groups (*cf.* cellulose, etc.).

Table XI shows the comparative distribution of the different side chain end groups in wool keratin and rabbit myosin, together with a proposed scheme of cross-linkage between opposed polar triads of the polypeptide grid (58). Figure 4 illustrates this scheme diagrammatically (58) within the framework of the  $\alpha$ -fold as depicted in Figure 3.

Figure 4 is a concrete example of what was meant above by our analogy between the

stoichiometry of the proteins and that of mixed crystals. And just as in a series of mixed crystals we generally have two "ideal" or limiting structures, one at either end of the series, so in effect Figure 4 postulates an ideal limiting keratin and an ideal limiting myosin. Of the polar cross-linkages in the limiting keratin, one-third are salt linkages, one-third amide-hydroxyl bridges, and one-third hydroxyl-hydroxyl bridges; whereas in the limiting myosin (from which rabbit myosin itself is not far removed), two-thirds are salt linkages and one-third are amide-hydroxyl bridges.\* It should be noted how this scheme gives expression to the ideal allocation of acids and amides put forward in Table IX. In this respect at least, there are good grounds for believing that what is aimed at is that keratin and myosin shall have the same numbers of asparagine and glutamine

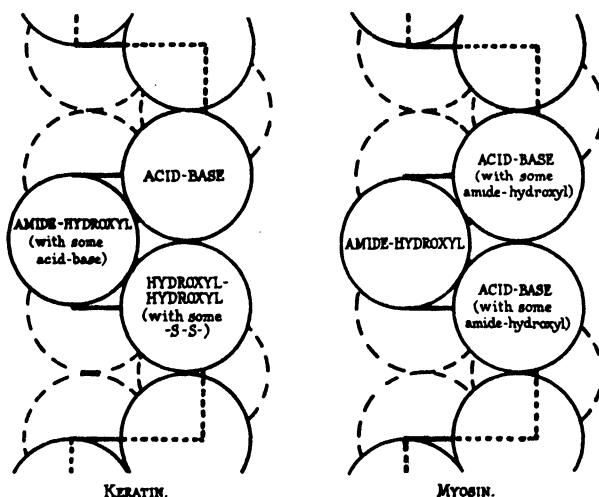


Fig. 4.—Proposed plan for the packing and cross-linkage of the polar side chains in keratin and myosin.

residues (24 and 32, respectively), while myosin shall have twice as many of both aspartic and glutamic acid residues (24 and 64) as keratin has (12 and 32).

There are two possible ways of realizing residue interchange (58): it may take place either within chains or between chains. In other words, the pattern of a single chain may be a sequence of certain *types* of residues allotted to definite sites—and in such a chain there will be a stoichiometry only of types, not of individual residues—or there may be a variable group-

\* For a more detailed discussion of this proposed stoichiometric scheme of cross-linkages in the keratin-myosin group, and, in particular, of the position of the sulfur bridges in the scheme, reference should be made to reference (58).

ing together of chains, or even of grids, of constant composition, with each chain or grid perhaps conforming strictly to the  $2^{\pi}3^m$  rule. On the whole, present evidence is probably more in favor of the latter concept, though it is quite reasonable, for all we know to the contrary, to suppose that both methods are used, according to circumstances. The evidence in favor of a grouping together of chains or grids comes from both chemical and crystallographic sources. Chemically, there are now known cases, such as lactoglobulin and egg albumin, to be discussed below, which require not a single chain but a number of chains for any completely satisfactory interpretation

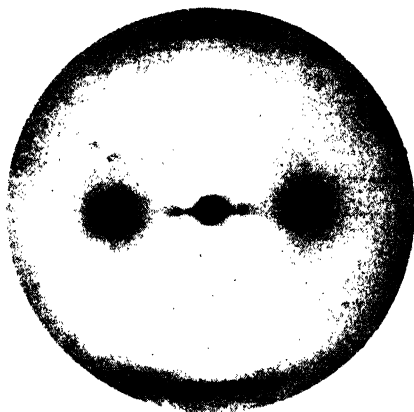


Fig. 5.—X-ray fiber photograph of the tip end of a porcupine quill, dried in a vacuum over phosphorus pentoxide. Cu K $\alpha$  rays. Collimator  $13 \times 0.025$  cm. (MacArthur.)

of their structure; while crystallographically, we have to reckon with the outstanding fact that the X-ray photographs of fibrous proteins, such as porcupine quill, feather keratin, collagen, etc., reveal large side spacings which show that the structural unit must be composed of a number of different, or differently oriented, chains lying side by side. This strongly reminds us of the plagioclase feldspars, built from continuously varying proportions of albite ( $\text{NaAlSi}_3\text{O}_8$ ) and anorthite ( $\text{CaAl}_2\text{Si}_2\text{O}_8$ ), and the recent X-ray interpretation of their structure in terms of a system of fine lamellae (84).

At the moment, the final elucidation of the structure of the keratin-myosin group looks most promising by way of porcupine quill, which gives much the best X-ray dif-

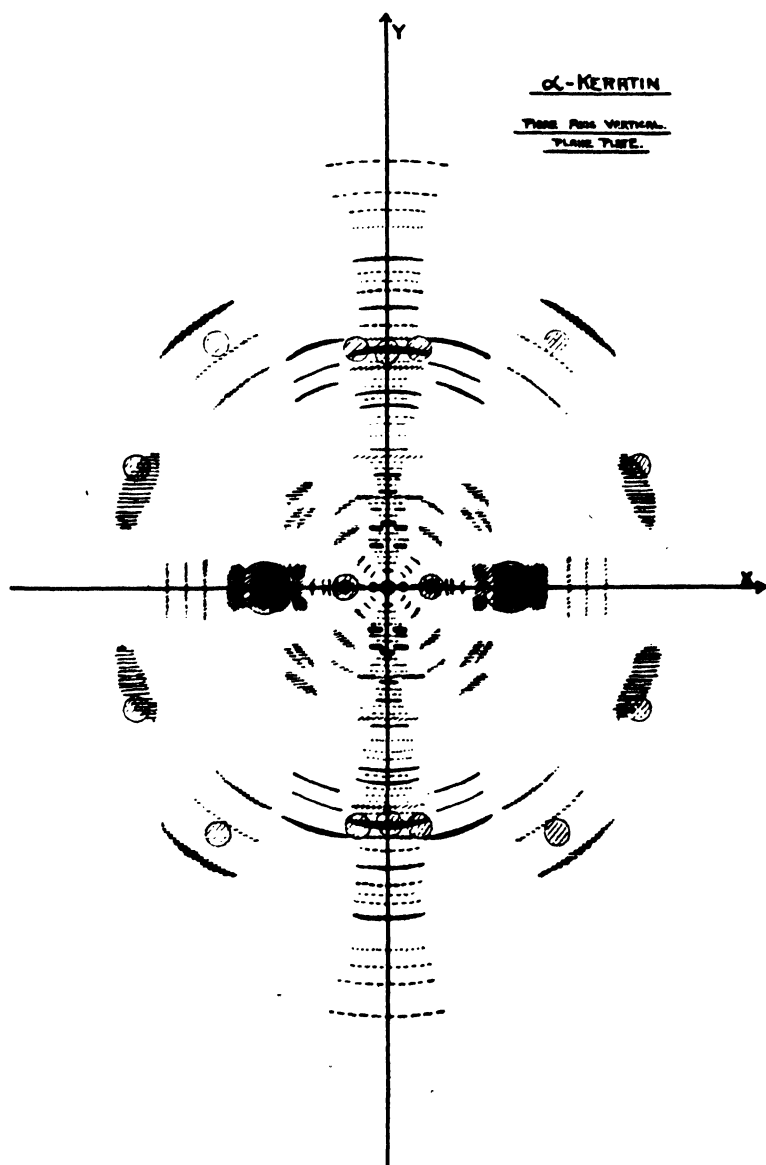


Fig. 6.—Composite diagram of the  $\alpha$ -keratin diffraction pattern given by porcupine quill (Cu  $K\alpha$  rays). The spacing of the most prominent arc on the meridian is 5.14 $\text{\AA}$ . (MacArthur).

fraction patterns of the series. Our earlier photographs of porcupine quill, though they indicated beyond doubt the presence of higher spacings, were like those of horn, for example, shown in Figure 1; but now MacArthur (85), working with greatly improved technique, has brought to light more than 100 reflections, ranging in spacing from 1 A. to 100 A., that together build up one of the most perfect protein fiber patterns yet encountered. Figure 5 is a reproduction of the central region of the porcupine quill photograph. The prominent group of reflections near the top and bottom corresponds to the arcs—the only arcs—seen in Figure 1, so it will be appreciated how very much more crystallographic information is now at our disposal. To emphasize this point, Figure 6 is a composite diagram that includes most of the  $\alpha$ -keratin pattern as we know it now. Given adequate chemical data, it should be possible to work out this pattern in considerable detail, and with such data as are available the task of interpretation is being proceeded with.

The molecular pattern along the fiber axis of porcupine quill repeats at probably 658 A. (this is a minimum distance to date), and the strong meridian reflection of spacing 5.14 A. that is so characteristic of the keratin-myosin group is the 128th (27th) order of that period. If 5.14 A. represents the length of an intramolecular fold comprising three residues, as we believe, then a single chain of the pattern-length revealed by X-rays would contain 384 (273) residues or a multiple thereof. Chemical analysis indicates 576 (293<sup>2</sup>), it will be recalled, but there is not necessarily any conflict, because both the X-ray and the chemical analytical numbers quoted must be considered for the present as the smallest compatible with the experimental data, and also because the chain may well be folded into long loops—it is easy, in fact, to suggest a type of loop that would conform with both numbers. The important thing to notice is that 384 is of the form  $2^3 3^m$ ; and moreover that *almost all the dominant reflections along the fiber axis are orders of 658 A. that are exactly or nearly of the form  $2^n 3^m$ .*

These reflections are tabulated in Table XII, due to MacArthur. In the upper half of the table we see that *all* meridian reflections of appreciable strength included between the center of the photograph and the reflection at 5.14 A. follow the rule closely, while in the lower half there are still seven other dominant reflections, of smaller spacing, that show a similar tendency (we should expect, in any case, the influence of the relative proportions of the residues to die out in reflections of smaller spacing, because the intensities of the latter will be decided more by the arrangement of atoms *within* the residues). It can hardly be argued that all this is pure coincidence, for in the crystallographic spacings listed there is none of the wide margin of experimental error overlapping hoped-for results that has been used as an argument against similar chemical agreements.

Here then, from a new and independent quarter, are the 2's and 3's again; and it is impossible to dismiss them lightly as an example of wishful thinking. What they signify in the case of  $\alpha$ -keratin is this, that the full molecular pattern is made up of smaller pseudo-patterns, just as that of the

tobacco mosaic virus is (86), and these pseudo-patterns subdivide the main pattern, sometimes more sharply, sometimes less sharply, into fractions of the form  $\frac{1}{2 \cdot 3^m}$ . It is doubtful whether this finding supports, for keratin and myosin at least, the Bergmann-Niemann theory (1) of the way the amino acid residues follow one another along the polypeptide chain, but it

TABLE XII (58)

DISTRIBUTION OF THE MAIN REFLECTIONS ALONG THE FIBER AXIS IN THE X-RAY DIFFRACTION PATTERN OF PORCUPINE QUILL ( $\alpha$ -KERATIN)

Intensity	Spacing (A.)	Order of 658 A.	Nearest $2 \cdot 3^m$
<i>All meridian, or very near meridian, reflections (d. <math>\geq</math> 5.1 A.) with intensity <math>\geq</math> W +</i>			
M	66.3	10	9
M	27.8 *	24	24
M	19.9,*	33	32
WM	18.10	36	36
W +	13.11	50	48
M -	12.35	53	54
W +	10.44	63	64
M -	6.16	107	108
Strong	5.14	128	128
Other dominant meridian reflections			
M	4.45	148	144
W -	4.00	164	162
W -	3.41 <sub>6</sub>	193	192
W +	3.08	214	216
v. W	2.55	258	256
(?)	2.29	287	288
M +	1.49	442	432

\* Layer line spacing. M = Moderately strong. W = Weak. v. W. = Very weak.

should be noted that it raises the status of column IV, Table VIII, to something higher than might be perceived through the gloom of stoichiopessimism.

## V. The Collagen Group (99)

Very few good chemical analyses are available for pure collagen fibers, most analyses having been carried out on gelatin, the protein derived from various collagenous structures by breakdown of a hydrolytic nature. The main features of the X-ray photographs of the collagen fibers and oriented



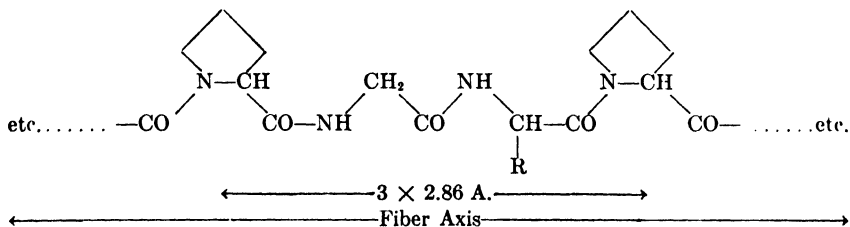
gelatin are always the same, but there are significant differences in detail, just as there are undoubted differences in chemical constitution: for this reason chemical analyses on gelatin are to be regarded as indicating probably only the main distribution of amino acid residues in the collagen fibers, though the same reason condones their use in trying to arrive at a general stoichiometric interpretation of the common features of the X-ray pattern.

There are many high spacings in the true collagen pattern (that of rat's tail tendon, for instance), but these have not been observed in the gelatin photograph, but only certain reflections characteristic of all collagen photographs. Most characteristic of all is the strong meridian arc of spacing about 2.86 Å., which we have identified with the approximate average residue length in the direction of the main chains. The side-chain spacing of the dry protein is 10.4 Å. at the most—at the most, because gelatin is difficult to dry completely, and then the X-ray pattern tends to disappear—but it is hard to be at all sure about the backbone spacing, because there is only a very diffuse reflection, of mean spacing about 4.4 Å., on this part of the equator. The dry density is about 1.32 gm./cc., and so if  $\beta$  be the angle between the side chain and backbone spacings and  $L$  the average residue length, then:

$$94 \times 1.65 = \frac{4.4 \times 10.4 \times L \times 1.32}{\sin \beta}$$

i. e.,  $L = 2.6 \sin \beta$  (approx.), which suggests that  $L$  cannot be greater than about 2.6 Å. It almost certainly is greater than 2.6 Å., though, and conceivably the error lies in the chosen backbone spacing, and perhaps even to some extent in the side-chain spacing also. Anyhow, there appears to be little doubt that the average residue length is considerably shorter than what has been found for the extended polypeptide chains in fibroin,  $\beta$ -keratin, etc., and this outstanding peculiarity of the collagen fibers must be linked in some way with their chemical constitution. Table XIII sets out the principal available chemical data for gelatin, together with two results for collagen proper. At present there is no convincing reason for departing seriously from the average residue weight ( $\bar{R} = 94$ ) chosen by Bergmann, or from the scheme of approximate frequencies put forward by Bergmann and the writer, and in any event there is no escaping the fact that closely one-third of the residues are glycine residues, while almost another third ( $5/18$  in the ideal frequency scheme) are proline or hydroxyproline residues. Also, as just explained, the X-ray diagram suggests an average residue length of the order of 2.86 Å. and a general grouping of

residues along the chains in sets of three—all of which considerations point strongly to a principal intramolecular theme of the form:



This is no more than the main theme, it must be emphasized, on which many variations are possible and are doubtless realized in nature: one way, for example, must be by virtue of the fact that once in every eighteen residues or so the imino ring is lacking, and the gaps are available for a variety of other residues. As in the keratin-myosin group, we have to explain the occurrence of a great family of structures, all with certain X-ray diffraction features and certain physicochemical properties in common, yet differing in chemical composition one from another; and just as in the keratin-myosin group a single master key may be found in an alternation of polar and nonpolar residues, so in the collagen group the key seems to lie in the proportions and preponderance of glycine and imino residues. It is unfortunate that analyses of various well-defined members of the collagen group are not available by which to check this all-important conclusion, but that is certainly the inference from the X-ray and chemical data. Over the whole field of the protein fibers X-ray analysis waits upon chemical analysis, and there is urgent need especially for complete chemical analyses of the actual specimens from which the best X-ray results have been obtained.

It follows from Table XIII that the number of residues in the molecular plan of the collagen group is (a) a multiple of 3; (b) probably a multiple of 72; (c) a number of the order of at least 200, to judge by the histidine, hydroxylysine and methionine contents; and (d) possibly of the order of 600, if the reported cystine content is reliable. The most likely minimum figure would appear to be 216 ( $2^3 3^3$ ), and that is also the number which accords best with the most recent X-ray results. Wyckoff and co-workers (101) reported the following meridian reflections from kangaroo tail tendon: 2.91 (s), 4.03 (w), 7.21 (w), 21.6 (v. w.), 24.1 (v. w.), 26.9 (w), 33.6 (m), 54.6 (s), 70.1 (s) and 103 (s); while we have observed a similar series from rat tail tendon (102), with certain significant intensity differences, such as 3.98 (m) and 9.5 (m). From results such as these, the chemical data, and a

reported direct measurement by Clark and co-workers (103) of a reflection at 432 Å., we concluded (52) that the full period along the fiber axis was a multiple of approximately 209.5 Å., and that the multiplying factor was

TABLE XIII

## GELATIN

$$R = '94, G = 1.06$$

Amino acid	Yield, %	Gram-residues		Proposed frequency
		Obs.	Calc.	
Glycine	25.5 (87)	0.3399	...	...
	25.5 (88)	0.3399	...	...
	26.5 (89)*	0.3533	0.3533	3 (3 <sup>1</sup> )
Alanine	8.7 (87)	0.0978	0.1178	9 (3 <sup>2</sup> )
Leucines	7.1 (87)	0.0542	...	...
	8.5 (23)	0.0649	0.0589	18 (2 <sup>1</sup> 3 <sup>2</sup> )
Phenylalanine	1.2 (90)	0.0073	...	...
	2.6 (91)	0.0158	...	...
Proline	19.7 (88)	0.1713	...	...
	17.5 (89)*	0.1522	0.1767	6 (2 <sup>1</sup> 3 <sup>1</sup> )
Hydroxylproline	14.1 (87)	0.1060	...	...
	14.4 (88)	0.1083	...	...
	14.7 (92)	0.1105	0.1178	9 (3 <sup>2</sup> )
Arginine	9.1 (93)	0.0523	...	...
	8.7 (65)	0.0500	0.0589	18 (2 <sup>1</sup> 3 <sup>2</sup> )
Lysine	5.9 (87)	0.0404	0.0442	24 (2 <sup>2</sup> 3 <sup>1</sup> )
Histidine	0.9 (87)	0.0058	...	...
Hydroxylysine	0.94 (94)	0.0057	...	...
Serine + hydroxylysine	3.3 (95)	0.0314	...	...
Threonine	1.4 (95)	0.0118	...	...
Tyrosine	0 (87)	...	...	...
Tryptophan	0 (87)	...	...	...
Cystine	0.2 (96)	0.0017	...	...
Methionine	1.0 (97)	0.0067	...	...
Aspartic acid	3.4 (87)	0.0256	...	...
Glutamic acid	5.8 (87)	0.0395	...	...
Amide N	0.33 (87)	0.0236	...	...
Total N	18.3 (98)	...	...	...

\* For collagen also.

possibly 4. It appears now, however, that there was some misunderstanding and that Clark and co-workers did not observe *directly* a reflection at 432 Å., but only inferred it; and it turns out from the latest direct measure-

ments by Bear (100) that the multiplying factor is actually 3 (or some multiple of 3, to be strictly correct). Bear, working with dried beef tendon, has recorded ten of the innermost orders of a fundamental period that he estimates to be about 640 Å. The over-all average from Bear's data and those of other workers is about 636 Å., and on this basis we have set out in Table XIV the full list of observed meridian reflections and their orders.

TABLE XIV  
COLLAGEN

Meridian reflections in the X-ray fiber diagram (Proposed period = 636 Å.)

Order	Spacing		Order	Spacing	
	Calc.	Obs.		Calc.	Obs.
1	636	...	19	33.5	33.6 <sup>b</sup>
2	318	322 <sup>a</sup>			32.2 <sup>c</sup>
3	212	217 <sup>a</sup>	24	26.5	26.9 <sup>b</sup>
4	157	161 <sup>a</sup>			26.2 <sup>c</sup>
6	106	106 <sup>a</sup>	27	23.6	24.1 <sup>b</sup>
		103 <sup>b</sup>			22.7 <sup>c</sup>
		?101 <sup>c</sup>	30	21.2	21.6 <sup>b</sup>
7	91	91 <sup>a</sup>	67	9.5	9.5 <sup>c</sup>
8	79.5	80 <sup>a</sup>	77	8.3	8.3 <sup>c</sup>
9	71	71 <sup>a</sup>	88	7.23	7.21 <sup>b</sup>
		70.1 <sup>b</sup>	139	4.58	4.59 <sup>c</sup>
		71 <sup>c</sup>	159	4.0	4.03 <sup>b</sup>
10	63.6	64 <sup>a</sup>			3.98 <sup>c</sup>
11	57.8	58 <sup>a</sup>	219-222	2.91-2.86	2.91 <sup>b</sup>
12	53	53 <sup>a</sup>			2.86 <sup>c</sup>
		54.6 <sup>b</sup>	238	2.67	2.67 <sup>c</sup>
		52.5 <sup>c</sup>	313	2.03	2.03 <sup>c</sup>

<sup>a</sup> Bear (100); <sup>b</sup> Wyckoff and Corey (101); <sup>c</sup> Astbury and Bell (102).

The equatorial reflections observed by Wyckoff and Corey from kangaroo tail tendon are: 5.42 (s), 10.9 (s), 19.9 (w), 30.0 (v. w.) and 47.6 (w); while our own measurements from rat tail tendon are: 3.6 (?), 4.4 (?), 5.62, 6.06 and 11.0 (at room humidity).

Another very recent and valuable investigation that lends support to the present conclusion that the fundamental period along the collagen chains is of the order of 636 Å. is that of Hall, Jakus and Schmitt (104), who have obtained electron microscope photographs ( $\times 25,000$ ) of single collagen fibrils from rat tail tendon, beef tendon and ligaments, human skin, etc. These photographs show a series of transverse bands of spacing that varies from about 520 Å. to 900 Å. according to the specimen. The X-ray spacings do not vary, however, so that what is revealed by the electron microscope, though

related in some way to the intramolecular period, is possibly a periodic distortion produced by intensive drying, a treatment that is already known to have an adverse effect on the intramolecular pattern along the fiber axis in gelatin. This combined investigation by means of the electron microscope and X-rays is being continued, and the outcome will be awaited with great interest.

Assuming, then, that the full period along the collagen chains is about 636 Å., a reflection at 2.86 Å. would be the 222nd order, and the 216th order would correspond to rather less than 3 Å. In other words, just such a minimum number of residues as might be inferred from the chemical analyses, built into chains of the kind proposed, might reasonably be expected to result in a period of the length observed, and could also very well reproduce the other characteristic features of the X-ray pattern. It must be confessed that there is an unsatisfying vagueness about this sort of statement, but the following points have to be kept in mind: (a) that we do not know, in view of what has been said earlier in this article, and pending complete analyses of individual collagens, to what extent the numbers of the different kinds of residues and the total number of residues in the molecule are governed *exactly* by the  $2^m3^n$  rule, though clearly there is a very strong tendency in that direction among the predominating residues; (b) that a maximum intensity among the meridian reflections need not occur exactly at a spacing representing the average residue length, though we might expect it to occur somewhere near; (c) that it is not certain whether the reflection at 2.86 Å. is a true meridian reflection, or whether it is really only a close layer-line reflection of spacing slightly less than the corresponding meridian translation; and (d) that the influence of the lateral grouping of chains is unknown. All things considered, therefore, it must be granted that the degree of co-ordination now appearing between the X-ray and chemical data is distinctly promising, and that in spite of an aggravating lack of quantitative precision the argument seems to be running definitely along the right lines. It should be noted, finally, that the interpretation of the collagen group outlined here remains essentially the same as that put forward (52, 99) before the appearance of Bear's results. The latter help to bring a much-needed precision to the X-ray side of the problem, but they do not introduce anything to compel a different point of view.

## VI. The Non-Fibrous or Corpuscular Proteins

**Insulin (105).**—Zinc insulin crystals are rhombohedral, space group R3, with one molecule per cell. Miss Crowfoot's data are as follows:

$$\text{Hexagonal axes} \begin{cases} \text{air-dried: } a = 74.8 \text{ Å., } c = 30.9 \text{ Å.} \\ \text{wet: } a = 83.0 \text{ Å., } c = 34.0 \text{ Å.} \end{cases}$$

Rhombohedral axes  $\left\{ \begin{array}{l} \text{air-dried: } a = 44.4 \text{ \AA}, \alpha = 114^\circ 28' \\ \text{wet: } a = 49.4 \text{ \AA}, \alpha = 114^\circ 16' \end{array} \right.$

Dry density = 1.31 gm./cc. Wet density = 1.28 gm./cc.

The calculated molecular weight in the dry crystals is 37,600,\* but in the wet crystals there is added to this a weight of liquid of crystallization equal to 14,800. There seems every reason to believe that the molecule in the dry crystals is the same thing as the Svedberg unit ( $M_s = 41,000$ ;  $M_v = 35,000$  (16)), and indeed the crystallographic evidence points to a similar state of affairs in the wet crystals too, except for a small rotation of the units about the *c*-axis and the penetration of liquid between them. The wet crystals give X-ray reflections down to about 2.4 Å. (1564 reflections have been recorded!), indicating a regularity of structure down to atomic

TABLE XV

## INSULIN

$$R = 123, G = 0.811$$

I—Percentage yield. II—Gram-residues in 100 gm. of protein. III—Observed frequency in 0.811. IV—Proposed frequency. V—Number of residues in 288.

Amino acid	I	II	III	IV	V
Arginine	3.05	0.0175	46.3	48 (2'3')	6
Histidine	10.7	0.0690	11.7	12 (2'3')	24
Lysine	1.26	0.0086	94	96 (2'3')	3
Cystine/2 (106)	12.5	0.1042	7.8	8 (2')	36
Tyrosine (107)	12.5	0.0691	11.7	12 (2'3')	24
(Amide NH <sub>2</sub> )	1.65	0.0971	8.4	...	34)

dimensions, and threefold symmetry in the diffraction pattern has also been observed down to this limit. Since there is only one molecule per unit cell, this probably means that each molecule is symmetrical about a triad axis, or very nearly so, though the chance of statistical symmetry must always be borne in mind for the present. The implication, therefore, is that the numbers of the different kinds of residues are all divisible by three, and this is substantiated at least for the acids quoted in Table XV, which is based on Chibnall's most recent results (14).

Chibnall finds  $G = 0.811$ , which gives an average residue weight of 123,

\* (Footnote added in proof): The most recent measurements indicate that the water-content of the air-dried crystals is in reality greater than the estimate hitherto used in the crystallographic calculations, and it now seems probable that the crystallographic molecular weight is about 35,600, in good agreement with the chemical value deduced by Chibnall (see below).

and he states that the accuracy of the histidine yield is probably 2% and that of the other results 3%. It seems pretty certain that the  $2^3 3^m$  rule holds for the acids quoted, and though the L.C.M. so far available is only 96 ( $2^3 3^1$ ), the independent evidence of the molecular weight points to a further multiplying factor of 3, giving 288 ( $2^3 3^2$ ) residues in the complete molecule. Using Chibnall's value of  $R$ , the molecular weight comes then to 35,500, approximately.

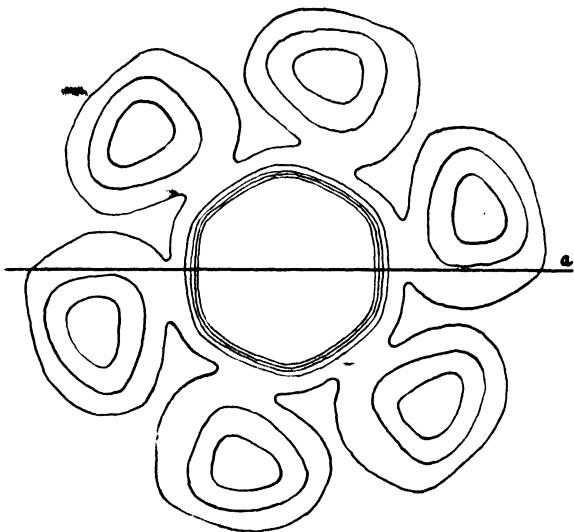


Fig. 7.—Wet zinc insulin. Patterson-Harker section parallel to (0001) at  $z = 0$  (Crowfoot).

Chibnall estimates by the Van Slyke manometric method that there are probably 18 free amino groups in the insulin molecule; that is to say, it is a system of 18 peptide chains. This finding is in remarkable agreement with a property, pointed out by Bernal (108), of the (0001) Patterson projection prepared by Miss Crowfoot from her X-ray data. To quote her own words: "In position all the observed peaks (in the projection) fall on a hexagonal network, the axes of which lie at an angle to the crystallographic axes. The angle observed is closely that required if the insulin molecule itself has a structure in which the eighteen points of the network around the origin are occupied by units which are arranged in a close-packed array, not only within one molecule but also with reference to the

unit structure of neighboring molecules. The change from dry to wet insulin then appears to involve an angular shift of the molecules from these close-packed positions. Further, the new peak positions in the wet (0001) projection are not far from a second hexagonal network, which might again bring the unit points into close contact" (109). There is nothing yet, however, in the crystallography to link the 18 peaks in the Patterson projection directly with the 18 peptide chains inferred by Chibnall and it would be unwarranted to jump to conclusions before the analysis now in progress has yielded its utmost, but obviously both X-rays and chemistry are here converging on something very fundamental in protein structure. And that something is undoubtedly the same thing as has shown itself from time to time in relation to the  $2^{\circ}3^{\circ}$  rule and throughout the whole of this stoichiometric discussion—we refer to the building of proteins from subunits whose make-up must be comparatively simple, and whose stoichiometry may conceivably be always a matter of 2's and 3's.

Another extremely interesting feature of the insulin Patterson diagrams is the occurrence of a number of high and well-defined peaks at about 5 Å. Figure 7 (reproduced here by courtesy of Dr. Crowfoot) is a Patterson-Harker section for wet zinc insulin parallel to (0001) at  $z = 0$ . Its appearance is striking, to say the least, and one cannot help wondering whether these strong mass-vectors (there are others, too, in other sections) may not ultimately be traced to an intramolecular source similar to that of the characteristic 5.1 Å. reflection of the keratin-myosin group; that is to say—if the present interpretation of the latter is sound—to a system of folded main chains and close-packed side chains. The time is not yet, though, to express any opinion of value, but the possibility is there and deserves to be examined most carefully. In the meantime X-ray progress with insulin will be followed with a keenness as great as any in protein studies.

**Lactoglobulin.**—According to Chibnall (14),  $\beta$ -lactoglobulin, though homogeneous by electrophoresis, ultracentrifuge and solubility tests, is definitely at variance with the  $2^{\circ}3^{\circ}$  rule. Table XVI has been drawn up by Chibnall from his own analytical results and some by Brand and Kassell (110). The average residue weight is estimated at 112.4, and the mean value of the molecular weight comes to about 42,000 (*cf.*  $M_r = 41,500$  and  $M_s = 38,000$  (16)). The numbers of residues for arginine and lysine are not factorisable by 2's or 3's, and neither is the estimated total number of residues (373). The deviations from the  $2^{\circ}3^{\circ}$  rule in the case of egg albumin might be explained on the ground of nonhomogeneity, as pointed out above, but there is no such escape with lactoglobulin: if there are structural anomalies, we must look for them inside the molecule itself.

As it happens, a solution is found at once simply by comparing the numbers of carboxyl and amino groups given by analysis with the corre-



sponding numbers given by titration (111). The reader is referred to Chibnall's Bakerian Lecture for a fuller discussion; here we need only state that while the number of carboxyl groups tallies, the number of free amino groups found by analysis is apparently 7 or 8 short of that found by titration and by the Van Slyke nitrous acid method. Chibnall infers from this that the lactoglobulin molecule is an association of 8, or more probably 9, peptide chains held together by linkages involving carboxyl but not amino groups; and if the inference is correct, as there is every reason at the moment to believe, then the  $2^{\circ}3^m$  rule need not hold for the complete molecule,

TABLE XVI

 $\beta$ -LACTOGLOBULIN

$$R = 112.4, G = 0.890$$

I—Percentage yield. II—Observed gram-residues in 100 gm. of protein. III—Frequency referred to 0.890. IV—Minimum molecular weight calculated from I and II. V—Assumed number of residues. VI—Calculated molecular weight of protein.

Amino acid	I	II	III	IV	V	VI
Arginine	2.89	0.0166	53.6	6,115	7	42,105
Histidine	1.54	0.0099	89.8	10,080	4	40,290
Lysine	9.75	0.0667	13.3	1,498	28	41,970
Tyrosine (110)	3.78	0.0209	41.7	4,790	9	43,110
Tryptophan (110)	1.94	0.0095	93.7	10,520	4	42,090
Methionine (110)	3.22	0.0216	41.3	4,633	9	41,700
Cystine (110)	2.29	0.0095	93.5	10,500	4	41,980
Cysteine (as $\frac{1}{2}$ cystine) (110)	1.10	0.0092	97.3	10,920	4	43,670
Glutamic acid	21.51	0.1463	6.1	684	62	42,400
Aspartic acid	9.88	0.0743	11.6	1,346	31	41,730
(Amide $\text{NH}_2$ )	1.29	0.0759	11.7	1,318	32	42,160

whether or not it holds for each individual chain. The evidence of egg albumin and lactoglobulin is invaluable: it is just the sort of thing that was wanted in order to make sense of the accumulating concordances and discrepancies; for surely there are enough of the former to demonstrate the existence of a  $2^{\circ}3^m$  rule of some kind or other, yet quite enough of the latter also to show that it cannot be of universal validity when whole molecules are in question. The most reasonable point of view now is that the rule holds for structural components, and hence sometimes for complete structures as well, with the corollary that the fewer the dissimilar components the closer the agreement that might be expected. Edestin, for example,

may well turn out to consist of only a few long chains,\* for there is not only the stoichiometric argument in favor of this, but also the pronounced fiber-forming properties of the seed globulins when denatured (50).

A great deal of X-ray work on lactoglobulin has been carried out by Riley (112), who has examined most thoroughly the various Patterson diagrams and other aspects of the matter. The outlook, though complex, is by no means unpromising. Riley has succeeded in disentangling the data to the extent of placing the molecules in the unit cell, deriving their probable shape, and suggesting possible combinations of intramolecular components, but unfortunately the analysis is not sufficiently advanced to permit of any unambiguous correlation with the stoichiometric findings. For further information the reader is referred to Riley's thesis and a forthcoming publication.

There are two varieties of lactoglobulin crystals, tabular and needle-shaped, and the following is a summary of the principal dimensional data:

	a	b	c	Density	Space group	Molecules per cell
	Tabular Orthorhombic					
Wet	67.5 A.	67.5 A.	154 A.	1.257	P2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub>	8
Partly wet	67.5	67.5	148.5	...	P2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub>	8
Dry	60	63	110	1.27	(P2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub> )	8
	Needle Tetragonal					
Wet	67.5	67.5	133.5	...	P4 <sub>2</sub>	8
Dry	56	56	(130)	(1.3)	...	8

There are no measurements of the water content of air-dried lactoglobulin crystals, and so far the X-ray data show only that the molecular weight is in the neighborhood of 40,000 to 41,000.

**Egg Albumin.**—On account of the incorporation in the egg albumin molecule of phosphoric acid and polysaccharide groups the interpretation of the acid- and base-binding data is as yet provisional. Nevertheless, because X-ray and other indications seem to point in the same direction as stoichiometry, it is well worth recording that Chibnall (14) surmises from the analytical and titration data that the molecule consists of at least four chains. The structure† proposed some years ago by the writer (114) was that of four superposed laminae separated by side chains. The suggestion

\* (Footnote added in proof): It has now been shown by Chibnall that the edestin molecule contains no more than one chain per weight of 50,000.

† That is, the structure of the *native* molecule. In the published summary (113) of Chibnall's Bakerian Lecture there is a misprint which says that the writer (W. T. A.) proposed a set of four laminae for the structure of the denatured molecule.

arose partly from X-ray studies of fibrous proteins and partly from an observation of Gorter's (115) that the area of the monolayer formed by the "spreading" of one molecule of egg albumin is approximately equal to the surface area of the native molecule, considered spherical. We may not argue that the sphere is hollow and so produces automatically a monolayer of the same area, because the density of undenatured is practically the same as that of denatured egg albumin (116): rather must we conclude that an apparent sphere of surface area  $4\pi r^2$  gives rise to four laminae each of area  $\pi r^2$ . And then as regards both density and dimensions the agreement is good, for the diameter of the egg albumin molecule as given by the ultracentrifuge is about 44 Å., while the thickness of a pile of four laminae would be four times the average protein side-chain spacing as given by X-rays, *i. e.*, about  $4 \times 10 = 40$  Å. Such a picture for egg albumin\* (and probably for other corpuscular proteins too) co-ordinates so many of the known facts (117) that it is particularly satisfactory to find that it is consistent also with the available stoichiometric evidence. Incidentally, it should be noted that the deviations from the  $2^*3^m$  rule reported for egg albumin by Chibnall could be explained on either intra- or intermolecular grounds, or both.

**Hemoglobin.**—A comprehensive X-ray examination of horse methemoglobin is being carried out by Perutz (118). The crystals are monoclinic, space group C2, with two molecules per cell, each molecule being symmetrical about a dyad axis. The cell dimensions at different stages of slow drying, starting from crystals suspended in concentrated solutions of ammonium sulfate, are as follows:

	<i>a</i>	<i>b</i>	<i>c</i>	$\beta$	<i>c</i> sin $\beta$
Wet	109 Å. $\pm$ 0.5	63.8 Å.	55.1 Å.	111.1°	51.4 Å.
Intermediate	109 $\pm$ 0.5	63.8	51.4	116.2	46.1
Intermediate	109 $\pm$ 0.5	63.8	53.5 $\pm$ 0.3	127.5 $\pm$ 0.5	42.3
Air-dried	104 $\pm$ 1.5	63.8	54.1 $\pm$ 1.0	137.5 $\pm$ 1	36.5

From such measurements and other observations that need not be described here it follows that the hemoglobin molecules form coherent sheets parallel to the *c*-plane, with layers of water and ammonium sulfate in between the sheets. On drying the crystals, the sheets move closer together and slide over one another, thereby increasing the monoclinic angle  $\beta$ . The hemoglobin molecule is a rigid unit unaffected by the degree of hydra-

\* A similar suggestion has more recently been put forward by Pauling (119).

tion of the crystal, and it is of the nature of a slab whose flat side is parallel to the *c*-plane. Its greatest length is parallel to its dyad axis (*b*-axis = 64 Å.), its thickness is about 36 Å. (perpendicular to the *c*-plane), and its width is probably about 48 Å. (in the direction of the *a*-axis). The optical properties indicate that there are more primary bonds parallel to the *a*-plane than to the other two principal planes, and also that the four haem groups lie parallel to the *a*-plane: in view of the dyad axis this would mean two haem groups on each *a*-face.

The analysis is proceeding, and with every hope of finding out much about the internal structure of the molecule. There is no correlation to be reported yet with stoichiometric data of the kind set out in Table III, but it is worth mentioning that about three-quarters of the side chains are non-polar. The nonpenetrability of the molecule by water would suggest that these side chains are used for internal linkages, leaving the remaining quarter, consisting of active side chains, to function on the periphery.

**Tobacco Mosaic Virus** (86).—The unit particles are rodlike bodies of such regularity of internal structure that it makes little difference whether we call them molecules or crystals. Their length is not easily estimated by X-ray methods, but it is at least 1500 Å. (120), and particles much longer than this are formed by aggregation. Their thickness is 150 Å., which means that the cross-section corresponds to the cross-section of only three crystallographic unit cells (hexagonal or pseudo-hexagonal). The unit cell dimensions are:  $a = 87$  Å.,  $c = 68$  Å.; and since the density is 1.34 (121), this is equivalent to a weight of 370,000. The sixth layer-line is very strong, however, and the reflections are markedly pseudo-rhombohedral in character, so the true repeat units may possibly weigh only one-sixth of this, *i. e.*, 62,000. The virus, like all viruses apparently, is a nucleoprotein, the conjugated nucleic acid being of the type of yeast nucleic acid (122) and present to the extent of 5% (123) or 6% (86). The phosphorus content (122) is given as 0.56%, and therefore the weight associated with one nucleotide is about 5500, which suggests, since the structure is hexagonal, that 12 nucleotides are associated with the X-ray unit estimated to weigh 62,000, whose volume is  $74,270 \text{ Å}^3$ . On this reckoning, the volume associated with one nucleotide is about  $6190 \text{ Å}^3$ .

Now the peculiar thing is this, that Bernal and Fankuchen conclude from a study of the strongest reflecting planes that the tobacco mosaic virus is based on roughly equidimensional subunits of side 11 Å., though clearly such subunits could be associated with only one-quarter of a nucleotide (or rather less), which at first sight seems stoichiometrically unreasonable. Perhaps the correct inference is that the function of each

nucleotide is to co-ordinate the proposed subunits in groups of four. The authors show, however, that there is also evidence for larger subunits, flat platelets of dimensions  $44 \times 44 \times 22$  Å., that are arranged in cubic close-packing. But the volume of each of these larger subunits would be associated with about 7 nucleotides, if we accept the various dimensional data just quoted, so here again, for the moment, we have what looks like a difficult number, both stoichiometrically and crystallographically.

The number of amino acid residues per nucleotide would appear to be something of the order of 50. It may be that the correct number is 48.

## VII. Summary

The chemical evidence is reviewed for a  $2^m 3^n$  rule governing the proportions of amino acid residues in protein structures. It is shown that under certain conditions such a rule manifests itself beyond doubt, but there are cases where it definitely does not hold. The conclusion is reached that the rule operates in the building of the constituent parts of protein structures, but to what extent it appears in the completed product is a question of the number of dissimilar components.

The fibrous proteins are classified according to the findings of X-ray analysis.

The chemical and X-ray evidence is reviewed on the structure of silk fibroin. The frequencies of the principal residues appear to be of the form  $2^n$ , but the existing X-ray data are inconsistent with the chemical data. The X-ray evidence to date does not support the view that the residues in silk fibroin follow one another always or exactly in the periods suggested by their relative proportions.

The chemical and X-ray evidence is reviewed on the structure of the keratin-myosin group of fibrous proteins. It is shown that the group must conform to a broad stoichiometry of *types* of residues incorporated in a common molecular plan. A model is proposed which co-ordinates the main properties of the group, both X-ray and chemical, which are then deducible from a single principle that, in general, polar and nonpolar side chains occur alternately along the polypeptide chains. It is doubtful whether the X-ray evidence supports the view that the residues are distributed along the chains in periods always corresponding to their relative proportions.

The chemical and X-ray evidence is reviewed on the structure of the collagen group of fibrous proteins. Again there is a common molecular plan in spite of variations in chemical constitution. The key appears to

lie in the preponderance and relative proportions of glycine and imino residues, for so far only on this basis are the X-ray data susceptible of a reasonable interpretation. Along the polypeptide chains both glycine and imino residues must repeat for the most part at intervals of three residues.

Chemical and X-ray evidence is reviewed that throws light on the stoichiometry and internal structure of insulin, lactoglobulin, egg albumin, hemoglobin and tobacco mosaic virus, and data are presented that satisfactorily correlate deviations from the  $2^{\circ}3^m$  rule with multiple components, both intermolecular and intramolecular.

### Acknowledgments

I should like to acknowledge my indebtedness and express my thanks to Dr. K. Bailey for helping me to collect the chemical data, and to Prof. A. C. Chibnall, F.R.S., Dr. Dorothy Crowfoot and Dr. I. MacArthur for kindly putting their valuable results at my disposal in advance of actual publication.

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# THE CHEMISTRY OF GLYCOGEN

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The term glycogen refers to a polysaccharide or, more properly, to a group of polysaccharides occurring in cell plasma, which yield glucose as the end product of hydrolysis and which give a red-brown, red or in rarer cases violet color reaction with iodine. Glycogen may occur in practically all animal cells as well as in yeast.

According to the definition comprised in the foregoing description, glycogen is not a pure chemical entity having characteristics independent of its origin but rather a biological conception. In the starch grains of many plant species, *e. g.*, in *Oryza sativa* var. *glutinosa*, are found carbohydrates which likewise hydrolyze to glucose and show a red color reaction with iodine. Products exhibiting a similar color reaction are also obtained by the decomposition of starch. Although these substances are certainly related to glycogen, as will appear later, it is not customary to designate them as "glycogens" and it seems advisable to follow the same practice in the present account rather than add to the confusion of terminology which already besets this domain of chemistry.

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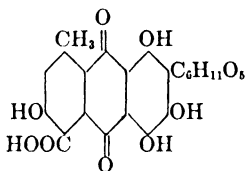
\* Translated by J. Edmund Woods, Queens College, New York.

## I. The Recognition and the Quantitative Determination of Glycogen

### 1. Qualitative Detection by Color Reactions

The presence of glycogen in cells can be detected by the red color produced by iodine-potassium iodide solution. The same reagent serves for microscopic testing of tissue sections. Inasmuch as certain other cell constituents also give a similar color, it is usual to immerse the section in saliva, whereupon the glycogen is dissolved, while other color-producing substances remain behind.

The coloration with carmine according to C. H. Best is also useful for microscopic detection. Carmine is an aluminum-calcium lake of carminic acid which occurs in cochineal solutions. The coloration disappears in the



presence of potassium chloride and ammonia. Glycogen becomes colored red.

### 2. Quantitative Determination of Glycogen in Organs

If the organ under investigation contains no other alkali-stable polysaccharides besides glycogen, the quantitative method introduced by Claude Bernard (6) and improved by Pflüger (55) can be used.

The pulped organ material is treated with an equal volume of hot 60% potassium hydroxide and precipitated with a like volume of alcohol. The mixture is washed, first with a mixture of one part 15% aqueous KOH and two parts alcohol and then with alcohol alone. The residue is then dissolved in water and hydrolyzed with HCl. The resulting glucose is determined by reduction (*e. g.*, by Bertrand's method). This is also the basis of the modern microchemical methods.

Soerensen and Haugaard (68) hydrolyze the glycogen with sulfuric acid and determine the glucose colorimetrically by means of orcin (methyl resorcinol).

Heatley (23) modified Pflüger's method so that as little as 1 mg.  $\pm$  2% can be determined. The glucose was determined by the procedure of Linderstroem-Lang.

Kerr (26) also devised a microchemical method which corresponds to Pflüger's macro method. It is especially suitable for the detection of very small amounts of glycogen in brain and nerve tissue.

Brand (7) invented a rapid micro method like that of Pflüger, the uniqueness of which depends upon adsorption of the polysaccharide by zinc hydroxide.

Sayun (63) in a comparable variation of Pflüger's method used animal charcoal as the adsorbing agent.

Simonovitz (66) criticized these adsorption methods and proposed the following method by which as little as 0.6 mg. of glycogen may be determined. Using the same conditions as those of Pflüger, he precipitated with alcohol in the presence of saturated sodium chloride solution. The precipitate was centrifuged out, dissolved in water, neutralized with sulfuric acid, filtered from the albuminous residue and then precipitated again by means of alcohol and saturated NaCl solution. The recovered glycogen was hydrolyzed with 2  $N$   $H_2SO_4$  and the glucose determined according to I. Bang.

By these methods, however, other polysaccharides which survive alkaline treatment and hydrolyze to reducing sugars are also determined. Among these, for example, is the mannan present in yeast. The salivary glands of snails (*Helix pomatia*), yielding galactose on reduction, respond to the same methods of determination. In such cases it is necessary to remove the other polysaccharides first; mannans and galactans are thrown out of the alkaline liquor by means of alkaline copper solution; the salts are thereupon separated by dialysis; finally the glycogen is precipitated with alcohol and determined as described above.

## II. Isolation

### 1. The Alkaline Treatment

In order to obtain the glycogen free from albumin or other cell constituents one had best follow the original instructions of Claude Bernard, treating the organ with strong alkali and precipitating with alcohol. The preparations obtainable commercially are always made by this method.

In the treatment with strong KOH, however, decomposition cannot be prevented unless oxygen is rigorously excluded. Accordingly, commercial glycogen has a considerably lower molecular weight than glycogen preparations which have been made with this precaution. Furthermore, in case peptides or lipoids are combined chemically with the cell proteins by primary valences, such groups will be split off during alkali treatment so that the analysis of glycogen obtained in this way tells nothing about the condition of the "native" glycogen.

In the following, therefore, we shall describe a few methods for the isolation of glycogen whereby the breaking of chemical linkages, as, for example, ester or amide linkages, is avoided; hence, by these means the glycogen can be obtained in its "native" condition. The glycogen obtained by such safeguarded procedures from muscle, liver and yeast is free from nitrogen and phosphorus. *Glycogen is therefore not united to protein or phosphoric acid by primary valences in its native condition.*

### 2. Isolation by Protective Methods

**Glycogen from Rabbit's Liver According to Bell and Young (5).**—The livers of several freshly slaughtered animals are placed in double their volume of boiling water, boiled

and at the same time disintegrated. The residue is ground with sand and extracted with boiling water repeatedly. The combined solutions are treated with one-tenth their volume of 40% trichloroacetic acid, allowed to stand at 0° C. for 15 to 18 hrs., centrifuged and then precipitated with 2½ times their volume of alcohol. Purification is achieved by precipitation with alcohol from aqueous solution containing some ammonium acetate or from water by addition of four times its volume of acetic acid. The drawbacks to this method are that not all the glycogen is likely to be extracted with hot water and, furthermore, that slight hydrolysis of the glycogen in the rather strongly acidic solution is not entirely prevented.

**Glycogen from Mussels (*Anodonta*)** According to K. H. Meyer and Jeanloz (39).—The muscular tissue of mussels (*Anodonta*) is broken down in a meat grinder and put immediately into an equal weight of boiling water. After centrifuging, the solution is precipitated with 1½ volumes of methyl alcohol, centrifuged again and dried with alcohol and ether. The residue is extracted in the same way repeatedly until the solution no longer gives a coloration with iodine-potassium iodide solution and is then extracted with 33% aqueous chloral hydrate for 60 min. at 80° C. Meanwhile the pH is kept between 6 and 7 by addition of sodium acetate. Precipitation is effected with methyl alcohol. By this treatment there is obtained a further glycogen fraction (Fraction IV) which is treated separately because of its albumin content and its low solubility. The residue is free from glycogen.

The crude glycogen is dissolved in water and the accompanying proteins precipitated with picric acid. The amount of picric acid must be regulated according to the amount of albumin present. For a 1% solution of muscle glycogen we use a third of its volume of 5% picric acid. The mixture is centrifuged and precipitated with 1½ volumes of methyl alcohol. Purification is accomplished by repeated precipitation and electro dialysis. The electro dialysis yields three fractions which can be separated from one another by careful siphoning: a soluble, clear fraction (I), a soluble, turbid fraction (II) and a precipitate (III). Their average nitrogen content is 0.6%. The three solutions or suspensions are treated separately according to Sevag (65), first with one-fifth their volume of chloroform, and then with one-fifteenth their volume of isoamyl alcohol. They are shaken for 10 to 15 hrs. and centrifuged for 1 hr. The proteins form a gel with the chloroform, leaving the glycogen in the aqueous solution. This procedure is repeated several times and then the glycogen is precipitated with alcohol. The nitrogen content is less than 0.06%.

The strongly albuminous glycogen, Fraction IV, consisting of about 80% protein and 20% glycogen, is extracted three times at 90° C. with five times its volume of 30% calcium chloride solution which has been brought to a pH of 7 to 8 by addition of magnesium carbonate. The bulk of the protein is left undissolved by this treatment. The extract is precipitated with 12% I-KI to which solid sodium chloride and some acetic acid have been added. The precipitate is centrifuged off, washed with 60% alcohol, decomposed with bisulfite, dialyzed and precipitated with alcohol. The glycogen so obtained forms a turbid solution in water.

**Preparation of Yeast Glycogen According to Stockhausen and Silbereisen (72).**—The yeast is ground up in a mortar with an equal weight of quartz sand and a fifth of its weight of diatomaceous earth. The mixture is put under hydraulic pressure of 350 atmospheres. A liquor is thus obtained which contains the glycogen, the mannans and the proteins. The yeast can also be treated under the same conditions in a mill having rotating steel rolls, afterward extracting with hot water (39). The mannans are sepa-

rated on adding an excess of Fehling's solution and allowing to stand for about 15 hrs. After filtration the solution is neutralized with acetic acid, dialyzed for 3 to 4 days with running water and electrodialed for 2 days. By repeated treatment with alcohol, glycogen and proteins are precipitated. The proteins are removed by Sevag's method (65).

### 3. *The Composition of Glycogen*

According to Taylor and McBride (74) and to Reich (59), the phosphorus as well as the nitrogen in glycogen are due to albumin which is mixed with it. Samec and Jsajewić (61) asserted that the readily soluble glycogen fraction obtained on electrodialedysis contained phosphorus. It is probable, however, that their solution retained phosphorus-bearing decomposition products of proteins or phosphatides. We ourselves found that the glycogen which had been very carefully prepared according to the foregoing directions and painstakingly freed of proteins contained less than 0.01% of phosphorus and less than 0.06% of nitrogen. From this one may infer that neither nitrogen nor phosphorus is chemically combined with glycogen. *Accordingly, glycogen contains no hexoseamino residues and no phosphorus.* The composition of pure, dry glycogen corresponds to the formula  $C_6H_{10}O_5$ .

## III. Fractionation; Properties of the Separate Fractions

Unfractionated glycogen preparations and also the commercial, partly decomposed products form opalescent or turbid solutions in water. The particles in glycogen solutions can be perceived by means of the ultra-microscope (E. Raehlmann (58)) and the electron microscope (Ruska and Husemann (60)).

Crude glycogen can be divided in various ways into fractions having different properties, namely: into those which dissolve in water to give clear solutions and those which give turbid suspensions and can be centrifuged. The separation can be accomplished by *Fractional precipitation*, by *electrodialysis* and by *rapid centrifuging*. The following illustrations may be cited:

From a commercial glycogen whose mean molecular weight they determined osmotically as 280,000 Staudinger and Husemann (70) obtained a cloudy, soluble fraction of molecular weight 800,000 by fractional precipitation of the aqueous solution with methyl alcohol.

Glycogen prepared by Pflüger's method can be divided into fractions of varying solubility by electrodialedysis, according to Samec (61). As explained previously, we applied this method to our refined glycogen. The

three fractions thus obtained retained their characteristics even after repeated precipitations. Fraction I gave a clear solution in water, Fraction II a turbid solution and Fraction III a suspension which could be centrifuged. The insoluble Fraction III dissolved in dilute sodium hydroxide. The solutions clouded up again on addition of acid. Chloral hydrate also brought about solution. In the cloudy solutions very large micells consisting of many single molecules were obviously present.

As will be seen further on, the viscosities of the acetates of the three fractions are different. This leads to the inference that they have different high molecular weights. The higher molecular fractions tend to associate and form very large supermolecular particles as the phenomena described above indicate. The high molecular, carefully prepared glycogen can be separated not only by electrodialysis but even by rapid centrifuging.

#### IV. The Molecular Weight

##### 1. *Osmotic Measurements in Water*

Aqueous solutions of glycogen contain molecular aggregates, as explained above. This behavior is exhibited also by aqueous solutions of amylose or of starch (30). Measurement of the osmotic pressure of aqueous solutions can give no information, therefore, about the weights of individual molecules.

On addition of alkali the solution clears up; yet the measured osmotic pressure of such solutions or of solutions in mixed solvents cannot be evaluated in any conclusive way. Indeed, the results obtained with any aqueous solution are never unequivocal but are highly inconsistent. Oakley and Young (48), using Oakley's high-pressure osmometer, measured the osmotic pressure in water and in 0.1 *N* calcium chloride solution of glycogen from rabbit liver, prepared according to Pflüger (55) or according to Bell and Young (5). The pressure in water was two to four times as high as it was in the salt solution, the former corresponding to a molecular weight of about 700,000 and the latter to a molecular weight of about  $2 \times 10^6$ . This difference can readily be explained by micell formation (association) under the salting-out influence of the calcium chloride. The authors, however, attributed it to ions; in water these unite, in consequence of the Donnan effect, to produce a higher pressure, whereas in salt solution the Donnan effect is repressed. If this was really the case, the glycogen used must have contained foreign, ionizable colloids. Staudinger and Husemann (70), using the relatively lower molecular weight commercial glycogen, which is probably broken down during manufacture, were unable to

detect any difference in the molecular weight (280,000) measured osmotically in water and in 0.1 *N* CaCl<sub>2</sub> solution. In formamide also the same molecular weight was obtained. Moreover, the acetylated product showed a corresponding weight (483,000) in chloroform. Again, in the decomposition products obtained upon acid hydrolysis they found agreement of weight in various solvents and among various derivatives. These results, however, reveal no information as to the condition of natural glycogen solutions where the question is one of considerably decomposed substances in which, obviously, the micell formation characteristic of glycogen in water falls off.

Oakley and Young have also measured the osmotic pressure of methylated glycogen from rabbit liver (methoxy content 45.1%) in 0.1 *N* calcium chloride solution and in benzene. They found molecular weights of  $1.3 \times 10^6$  in calcium chloride and of  $3.4 \times 10^6$  in benzene. Neither the salt solution nor benzene, in which substances having polar groups ( $-\text{OH}$  or even  $-\text{OCH}_3$ ) associate, are free from objections, however, as solvents for such determinations.

## 2. *Osmotic Measurements on Glycogen Derivatives*

As a unique method free from objection there remains the conversion of glycogen into derivatives of the same degree of polymerization and the measurement of osmotic pressure in such solvents as solvate the groups of derivatives. As soluble derivatives the acetyl and the methyl compounds come under consideration. They can be prepared easily without decomposition. Methylation, which is conducted in an alkaline medium, however, must be accomplished with strictest exclusion of oxygen if decomposition is to be avoided. Carter and Record (8) investigated methylated and acetylated glycogens of various origins in a number of organic solvents ( $\text{CH}_3\text{Cl}$ ,  $\text{CCl}_4$ ,  $\text{CH}_3\text{NO}_2$ ) and obtained values of 273,000 and 830,000 for the methylated products in chloroform. The acetylated products, also measured in chloroform, gave values of  $1.9 \times 10^6$  to  $3.6 \times 10^6$ . The disparity between these two sets of values may be traced back to a breakdown during methylation.

Van der Wyk and Jeanloz (77) found for an acetyl glycogen prepared with exceptional care and examined in benzyl alcohol a lower limiting molecular weight of about  $5 \times 10^6$ . The derivative had been made from the readily soluble Fraction I of muscle glycogen by treatment with pyridine-acetic anhydride. The less-soluble fractions tended to show molecular weights considerably higher still. Benzyl alcohol was selected as the solvent because it contains polar as well as apolar groups, so that it can solvate



both kinds of groups of the dispersed material. Micell formation, which is due principally to a closing up of polar groups in apolar solvents or of apolar groups in polar solvents, is thereby prevented.

From all the recorded observations it appears that undecomposed glycogen of various origins possesses a molecular weight in excess of four million. The difficultly soluble fractions very probably have weights amounting to several times this figure.

## V. The Shape and Size of the Glycogen Molecule

### 1. *Osmotic Phenomena and Molecular Shape*

It is a well-known characteristic of very dilute solutions that the magnitudes of certain properties such as osmotic pressure and viscosity are proportional to the concentration of the solute. These relationships are explained by the circumstance that the dissolved particles are completely saturated with solvent so that mutual effects among the particles themselves do not occur. This condition would be strictly fulfilled only at zero concentration, for at any finite concentration a certain proportion of solute molecules are not surrounded solely by solvent molecules but may encounter other solute molecules.

Without elaborating upon steric considerations at this time, it is clear that the probability of collision in a dilute suspension of spherical particles should be much less than it would be in a suspension of the same concentration of fibrous molecules. We find, therefore, in solutions of high polymers with compact molecules conditions similar to those prevailing in solutions of lower molecular compounds.

Until now, x-ray analysis of crystalline compounds, as well as examination by the ultracentrifuge, have indicated a compact, nonfibrous molecular structure for proteins. For example, from the osmotic pressure and the specific viscosity (40) of hemoglobin and many other proteins it can be recognized that the same simple relationships obtain here as among lower molecular compounds. Both the  $\pi/c$  quotient (osmotic pressure/concentration), also called the reduced osmotic pressure, and the  $\eta_{sp}/c$  quotient (specific viscosity/concentration) are constant up to several per cent.

Chain polymers, on the other hand, with a fibrillar structure to their molecules, show considerable deviation from this simple behavior. These deviations depend upon the circumstance that filament-shaped molecules are not completely surrounded by solvent even in very dilute solution but are continually encountering other solute molecules (17). This goes without saying, if we recall that a fibrillar molecule must have far more

neighboring particles than a spherical particle would have and that the probability of such a molecule's encountering parts of another dissolved chain molecule are correspondingly increased. Accordingly, we are able to draw inferences as to particle shape from behavior of the  $\pi/c$  quotient.

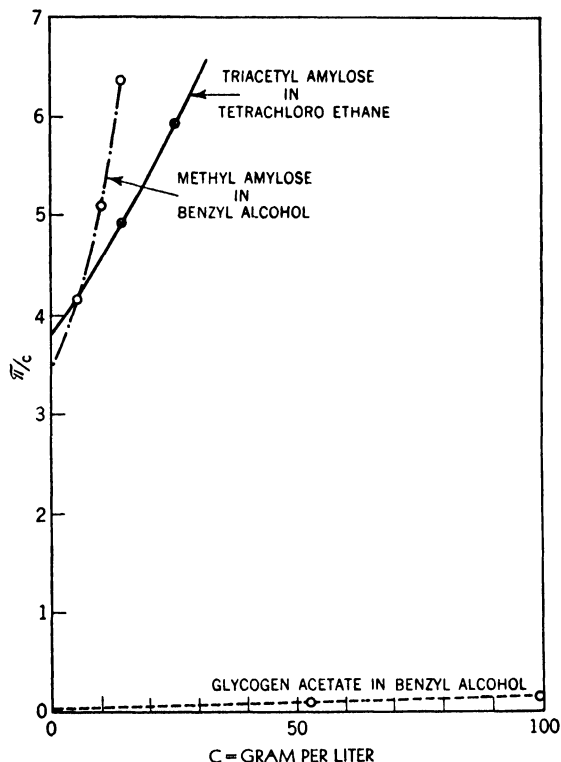


Fig. 1.—Reduced osmotic pressure ( $\pi/c$ ) at 25°C.  $\pi$  in mm.  $H_2O$ ;  $c$  in g. per l. solution.

Whereas certain compounds closely related chemically to the acetate of glycogen, *e. g.*, the acetate of amylose or of cellulose, show this deviation from the normal van't Hoff relationship, glycogen acetate does not do so; its  $\pi/c$  ratio is practically constant.

Figure 1 gives the  $\pi/c - c$  curves of methyl amylose, amylose acetate and glycogen acetate (Fraction I of muscel glycogen). Glycogen acetate evidently does not have a filament-like structure, whereas amylose acetate molecules deviate very considerably from the spherical form.

Staudinger and Husemann (70) had already noted the constancy of  $\pi/c$  for partially decomposed glycogen in 1937 and from this as well as from its viscosimetric behavior had ascribed a spherical shape to the glycogen molecule.

## 2. Viscosimetric Behavior and Molecular Weight

As already explained, for solutions of spherical particles, the specific viscosity in the dilute range is proportional to the concentration. Chain polymers of high molecular weight, on the other hand, exhibit deviations from this proportionality even in highly dilute solutions. This distinction permits inferences to be drawn regarding particle shape. Far more useful in this respect, however, are the absolute values of specific viscosity. For suspensions of spherical particles this property has definite, theoretically calculable values. Departures from sphericity cause the specific viscosity to exceed the theoretical value many-fold. For spherical molecules the shape does not change with the molecular weight; the specific viscosity should, therefore, be independent of the molecular weight. For chain polymers the deviation from sphericity increases with increasing molecular weight; hence a dependence of viscosity upon molecular weight is to be expected. From hydrodynamic considerations Einstein derived the following relationship for a solution of spherical molecules whose size is very large compared with that of the solvent molecules:

$$\eta_{sp.} = 2.5 \varphi$$

where

$$= \frac{\text{Volume of suspended particles}}{\text{Volume of suspension}}$$

As is apparent from this equation, the viscosity is independent of the size of the molecules (molecular weight or molecular volume). Polymers of different high molecular weights should show equal viscosities at equal concentrations, provided their particles are spherical in shape. This is the case for glycogen, according to Staudinger and his co-workers (70, 71). For glycogen and glycogen derivatives of various molecular weights dissolved in formamide, they found the following specific viscosities:

Mol. wt.	1 g. glycogen $\eta_{sp.}$ /100 cc. soln.	1 cc. glycogen $\eta_{sp.}$ /100 cc. soln.*
800,000	0.086	0.12
280,000	0.086	0.12
66,000	0.083	0.12

\* The calculation of these values is based upon a density of 1.3 for glycogen.

A solution of spherical particles in a 1% solution (by volume) should have a specific viscosity of 0.025 according to the Einstein equation. The value four times this figure signifies, according to Staudinger, that the glycogen is surrounded by a solvate shell. Staudinger and Husemann (70) obtained values similar to those in the table for glycogen triacetate in chloroform.

K. H. Meyer and Jeanloz (39) obtained the following specific viscosities for the acetates of the three glycogen fractions described earlier, all dissolved in tetrachloroethane:

Fraction	Mol. wt.	1 g./100 cc.	2 g./100 cc.	4 g./100 cc.	1 cc./100 cc.*
I	$5 \times 10^6$	0.09	0.19	0.44	0.12
II	$>5 \times 10^6$	0.11	0.24	0.53	0.14
III	$\gg 5 \times 10^6$	0.17	0.36	0.89	0.22

\* The calculation of these values is based upon a density of 1.3 for glycogen.

Accordingly, a dependence of viscosity upon molecular weight is evident in native glycogen, which signifies a deviation from spherical form.

### 3. *The Size of the Glycogen Molecule*

It may be assumed that the individual molecules of native glycogen exceed 100 Å in each dimension; for, assuming a density of 1.3, spherical molecules having a molecular weight of  $5 \times 10^6$  would have a diameter of 250 Å.

## VI. The Methods of Determining Constitution of Polysaccharides of the Starch Group

### 1. *Comparison between Glycogen and Starch*

The end product of hydrolysis of glycogen is glucose. Attack by  $\beta$ -amylase yields considerable amounts of maltose. Accordingly, the  $\alpha$ -1,4 glucosidal linkage through which the two glucose units in maltose are united certainly occurs plentifully in the glycogen molecule also. The specific rotation of glycogen in water is a value similar to that of starch. From this it may be concluded that, just as in the case of starch, the union of glucose units is mainly or entirely a question of  $\alpha$ -glucosidal linkages.

Notwithstanding all its resemblances to starch, however, there are important differences between glycogen and the starch polysaccharides.

The color reaction with iodine is different. Furthermore, starch is crystalline, as x-ray analysis shows. It even solidifies from solution in crystalline form. Glycogen, on the contrary, is amorphous. Finally, as already explained, the osmotic and the viscosimetric behavior of the two substances differ. All these differences are due to constitutional dissimilarities which are explainable by methods that have proved reliable in starch chemistry. For that reason, they should be reported next.

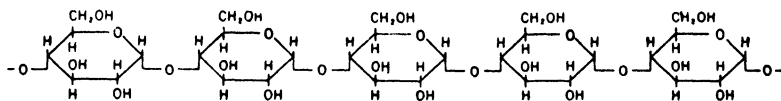
## 2. *Methods of Determining Constitution by Methylation and Cleavage*

The most important method for establishing the constitution of polysaccharides is the one taken over from the chemistry of oligosaccharides, which consists of methylation followed by hydrolytic cleavage and subsequent study of the methylated monosaccharides. Those hydroxyl groups of a hexose residue which were united glucosidally with other residues in the polysaccharide do not methylate but remain in the split sugar as free hydroxyl groups. If, for example, 2,3,6 trimethylglucose is found, these units were bound in the 1 and 4 positions with other residues; on the other hand, 2,3,4,6 tetramethylglucose, in which all alcoholic hydroxyl groups are occupied, would only have been joined to the aldehyde group (atom 1) of another glucose unit; accordingly, it derives from a terminal group. The number of tetramethyl units divided by the total number of units indicates the proportion of terminal groups. Dimethylglucose, on the other hand, comes from units which were united to the aldehyde groups of two other sugars; hence, it derives from branch points or, alternatively, from incompletely methylated links of the chain. If  $n$  is the number of terminal groups per molecule, the molecule has  $n-1$  branch points; in large, multi-branched molecules the terminal group content and the junction content are practically the same. If, in addition, one knows the degree of polymerization,  $P$ , from osmotic measurements, *i. e.*, the number of monomers per molecule, he can then discover from the terminal group content whether or not the molecule is branched. In unbranched chains the terminal group content equals  $1/P$ .

This method, therefore, furnishes information about terminal groups and branch points. For the characterization of various substances we propose to introduce the expression *Degree of Branching*, which will specify the number of branch points per total number of glucose units.

Application of the foregoing method to the polysaccharide fractions obtained from starch by fractional extraction with warm water showed that most starches contain about 10% of an unbranched polysaccharide

with  $\alpha$ -1:4 glucosidal linkages; this is designated as amylose (K. H. Meyer, Bernfeld and Wertheim (35, 36)).



Section of Amylose Chain.

The amylose fraction can be split up into fractions having molecular weights between  $10^4$  and  $10^6$  (34). The bulk of the starch, however—the paste-forming amylopectin—consists of a mixture of branched polysaccharides whose degree of branching is between 0.03 and 0.05.

### 3. Establishment of Constitution by Enzymatic Breakdown

The method of methylation reveals the degree of branching but does not indicate the arrangement of the branches. The clarification of this matter is aided by investigating the breakdown effected by  $\beta$ -amylase, as K. H. Meyer and Bernfeld (31) showed.

In consequence of the work of Ohlsson (49), Myrbäck (45), Hanes (18) and K. H. Meyer (31, 37), we know that the  $\beta$ -amylase of grain splits maltose from the nonaldehydic end of starch polysaccharides. At the beginning of the enzyme action maltose forms almost immediately, while the high molecular weight of the starch is changed only negligibly. Amylose, which consists of unbranched maltose chains, is completely broken down by amylase (Samec and Waldschmidt-Leitz (62)). On the other hand, amylopectin, which is branched and contains branch linkages as well as 1,4 linkages—indeed, it contains 1,6 linkages besides, as Freudenberg (15) has shown—is decomposed into a high molecular residue (residual dextrin). Accordingly, one can assume, as Myrbäck (45) does, that the junctions are obstacles to enzymatic action.

Thus,  $\beta$ -amylase splits off only the exterior branches. From the number of resulting maltose units, which can easily be determined by any reduction method, one can discover what proportion of the glucose units occurred in the exterior branches. The branch points can now be attacked by another enzyme; by this time other branches are available consisting of glucose units in  $\alpha$ -1,4 union, which again can be split off by  $\beta$ -amylase until a junction once more obstructs the decomposition. In this manner one can obtain a very good insight into the architecture of the amylopectin molecule.

#### 4. Qualitative Analysis with the Aid of the Iodine Color Reaction

The color which a polysaccharide gives with iodine, as the following table shows, is dependent, first of all, upon the degree of branching. For the sake of better comparison the results obtained with glycogen and its residual dextrin are included.

Substance	Degree of branching	Color reaction in iodine
Amylose	0	Pure blue
Amylopectin	0.04	Blue-violet
Starch from rice gluten	0.06	Red-violet
Residual dextrin from maize	0.09	Red
Glycogen (Merck)	0.09	Brown-red
Residual dextrin from glycogen	0.18	Light brown

Strongly decomposed amyloses obviously give a red reaction also. A blue reaction, however, is found *only* among unbranched or very slightly branched starch polysaccharides. If, therefore, a polysaccharide of this group gives a blue color with iodine, it is evidently unbranched or very slightly branched. If it gives a red iodine reaction and is to be regarded as a high molecular substance (molecular weight > 5000), its degree of branching is certainly greater than 0.04.

These qualitative methods will play a role in the discussion of synthetic "glycogens" (see page 131).

## VII. Determination of the Constitution of Glycogen

### 1. Result of Methylation and Cleavage

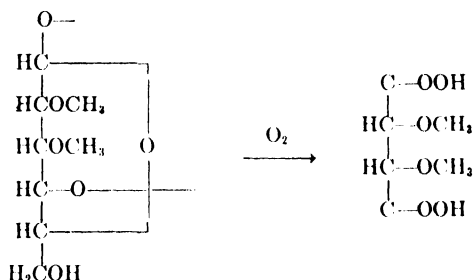
Glycogen possesses a ramified structure, as was first recognized by Haworth, Hirst and Isherwood (21). They methylated glycogen with dimethyl sulfate in alkaline solution, hydrolyzed, converted the methyl sugar into the methyl glucoside and separated the mixture of glucosides by fractional distillation. No sharp separation of sugars is obtained by this method but the content in the several fractions of tetramethylglucoside, which could come only from the nonaldehydic terminal glucose units, and of trimethylglucoside can be determined from their refractive indices, for the refractive indices of tri- and tetramethylglucosides are known (Hirst and Young (24)). An example of such analysis is given in the following table:

## METHYLATED GLYCOGEN (MERCK'S)—METHOXY CONTENT, 42% (38)

Fraction	Bath temp., C.	Pressure in mm. Hg	Weight, gm.	$n_D^{20}$	Tetra- methyl- glucose, %	Weight of tetramethyl- glucose, gm.
1	95-98	0.01	0.330	1.4447	98.4	0.324
2	115-118	..	0.130	1.4499	56.8	0.073
3	123-128	..	0.395	1.4560	8.0	0.032
4	123-128	..	0.335	1.4570	..	...
5	125-128	..	0.500	1.4572	..	...
6	130-135	..	0.245	1.4583	..	...
7	135-140	..	0.520	1.4586	..	...
8	140-150	..	0.685	1.4597	..	...
9	170-200	..	1.500	....	..	...
10	Residue	..	0.30	....	..	...
			4.940			0.429

Allowing for the losses suffered during the operations, the results indicate an end group content of  $9 \pm 1\%$  and a degree of branching of  $0.09 \pm 0.01$ .

Hydrolysis also produces dimethyl glucose, which is derived from the branch points. Haworth, Hirst and Isherwood were able to obtain dimethyl tartaric acid from this dimethyl glucose by oxidation; this proved its constitution to be that of 2,3-dimethylglucose.



The glucose residues from which this dimethyl glucose was derived were therefore glucosidified in positions 4 and 6; hence the "branch" occurs at position 6. Haworth, Hirst and Isherwood (21) concluded that all branches occurred at position 6 (see diagram). To be sure they left the question unsettled as to whether the union is really a true glucosidic linkage or, perhaps, a linkage of unknown kind. Then in a later paper (22) they adopted the opinion of other investigators that this is a case of true  $\alpha$ -1:6 glucosidic linkage.

The results of Haworth, Hirst and Isherwood did not, as yet, warrant



the positive exclusion of glucosidic linkages other than the  $\alpha$ -1:4 and the  $\alpha$ -1:6 linkages. For this purpose an investigation was necessary to show that the number of terminal groups (and therewith the number of branch points also) was equal to the number of 1,6 bonds.

Shortly after Haworth, Hirst and Isherwood had done so, Staudinger and Husemann (70) also came out in favor of a ramified structure for glycogen. From the remarkably low viscosity of glycogen and its osmotic properties they concluded, as already noted in this account, that glycogen molecules have a spherical contour. This they explained constitutionally by extensive branching and that the glucose units of a principal chain containing up to 100 units should have all three hydroxyl groups united glucosidally with branches consisting of 12 to 18 glucose units apiece. If this formula were correct, glucose would have to appear among the sugars split off from methylated glycogen, as Parnas (52) among others pointed out, and that is not the case. Neither can the formula be reconciled with the results of decomposition by  $\beta$ -amylase. For these reasons the constitutional formula submitted by Staudinger and Husemann is unacceptable.

According to Haworth and his co-workers (21, 22), glycogens can have varying proportions of terminal groups. That is, they are highly ramified but to different extents. Two different samples of rabbit liver contained 7% and 9% of terminal groups, respectively (degree of branching 0.07 and 0.09).

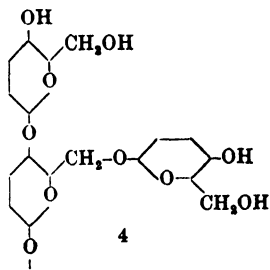
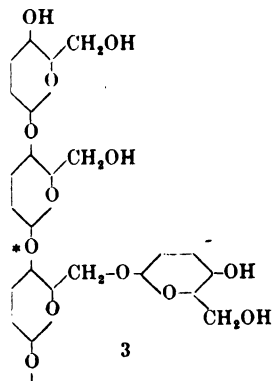
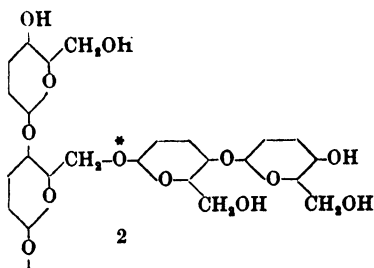
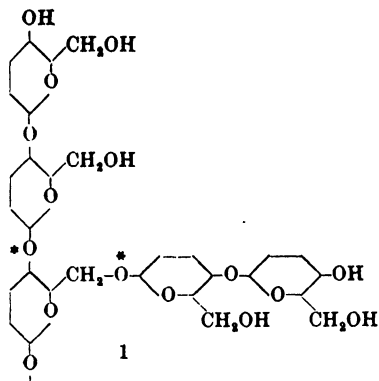
Claude Bernard (6) reported that long-dormant muscles yield a glycogen which is colored blue with iodine. This glycogen must, therefore, have a much less ramified constitution than ordinary glycogen (see page 122).

## 2. Results of Enzymatic Breakdown

Just as in the case of starch, one can obtain a deeper insight into the architecture of the glycogen molecule by decomposition with  $\beta$ -amylase.

Glycogen, like amylopectin, is broken down by  $\beta$ -amylase to give maltose and a high molecular residual dextrin. The latter substance, according to K. H. Meyer and Fuld (38), still contains all terminal groups—the groups with free hydroxyls in 2,3,4,6 positions—that were present in the original material. The glycogen used for this study (Merck's) contained 9% of terminal groups, *i. e.*, 1 terminal group for every 11 glucose units. By the action of  $\beta$ -amylase 47% was broken down. The residual dextrin which resisted  $\beta$ -amylase and was left behind, making up 53% of the glycogen, contained 18 terminal groups, *i. e.*, 1 terminal group to every

5.5 glucose units. From this one can arrive at a conclusion about the disposition of the branch ends which were not attacked by  $\beta$ -amylase. They must conform to one of the following four formulas, since each branch must still possess the end group.



In the residual substance there are now 5.5 glucose units to 1 terminal group. Of these, 1.5 glucose units on the average form the end groups along with branch ends so that only 3 glucose units are left over for the interior of the chains. When the glycogen is intact, there is also exterior branches present containing 6 to 7 glucose units apiece (see Fig. 2).

The residual substance (Residual dextrin I) of glycogen can now be attacked in the presence of phosphates by phosphorylase, which occurs in

the juice obtained on macerating yeast (see page 127); this leaves a second residue which can be freshly attacked by  $\beta$ -amylase. The only explanation for this is that the branch points are fractured by the phosphorylase, whereupon the chain fragments so exposed are no longer protected against amylase (33).

In the light of the foregoing presentation we can summarize the differences between glycogen and starch as follows: starch is a polymeric, homologous mixture of unbranched (amylose) and of branched (amylopectin) molecules. Glycogen contains only branched molecules of very high molecular weight. The degree of branching of amylopectin amounts at most to about 0.04 and that of glycogen at most to 0.09. The exterior branches of amylopectin consist of 15 to 18 glucose units and the inner

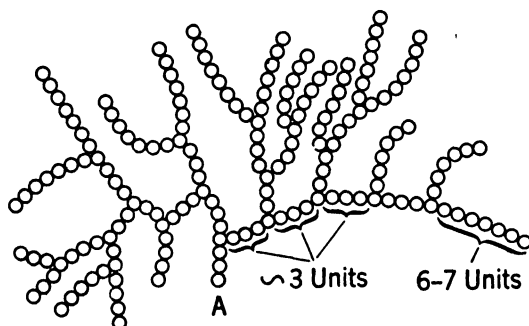


Fig. 2.—Structure of glycogen. A, aldehydic end.  
O, glucose units.

parts of the chains lying between the branch points consist, on the average, of 8 to 9 glucose units. The outermost spurs of the glycogen molecule are 6 to 7 units long. In the interior between branch points there are short chain fragments averaging 3 glucose units. Hence, glycogen is much more compactly built. This structure explains the inability of glycogen to form primary valence chain lattices and accounts also for its viscosimetric and osmotic differences from starch.

## VIII. The Biological Breakdown

### 1. Breakdown in the Digestive Tract

Glycogen which is taken in with nutriment is very probably broken down completely into glucose by the amylases of the saliva and the pan-

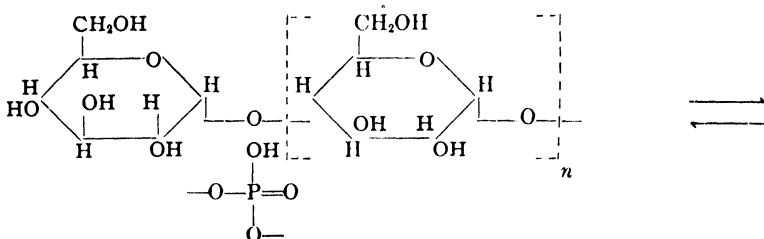
creas and also by glucosidases, *e. g.*, the  $\alpha$ -glucosidase of the intestines. Maltose, however, is not the exclusive intermediate product of decomposition, as has been incorrectly assumed. To be sure, maltose is found among the products of glycogen breakdown by pancreatic amylase but always mixed with oligosaccharides and with glucose. Determination of reducing power and of rotation are not sufficient for analysis, as Smits van Waesberghe (67), among others, emphasized. A mixture of maltose, reducing dextrans (tri- and tetrasaccharides) and a corresponding amount of glucose can exhibit the same reducing power and the same optical rotation as pure maltose. Dextrans, maltose and glucose must therefore be determined separately. This is best accomplished by selective fermentation, for example, by the method described by Somogyi (69) as follows: at a pH of 8.4 only glucose is fermented by baker's yeast; in unbuffered, neutral solution glucose and maltose are fermented. For estimation of the chain length of the dextrans remaining behind their reducing power before and after acid hydrolysis is determined. The quotient:

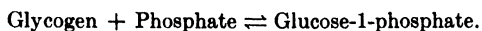
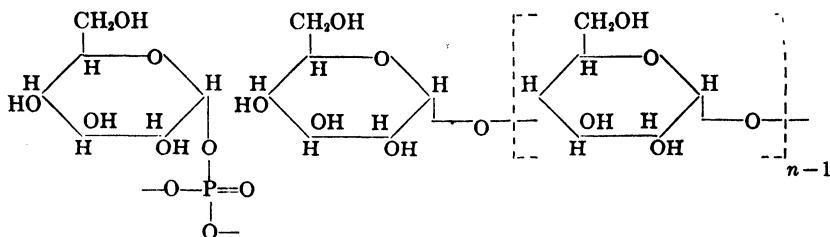
$$\frac{\text{Reducing power after hydrolysis}}{\text{Reducing power before hydrolysis}}$$

gives the degree of polymerization (in this connection see K. H. Meyer and Bernfeld (32)).

## 2. The Phosphorolysis of Glycogen

The breakdown of glycogen which has been stored up in individual organisms proceeds entirely differently. The first step is phosphorolysis, the reaction between glycogen and phosphate to yield hexose phosphates, which was discovered by Parnas (53) and has recently been studied intensively by Cori (9-14), Kiessling (27), Schäffner (64) and others. The first transition product is the glucose-1-phosphoric acid ester discovered by Cori (9):





Enzymes which catalyze this reaction are found in muscle, liver and other animal organs, likewise in yeast and in many parts of higher plants. They are designated as phosphorylases.

Cori (10) and his co-workers prepared phosphorylase from various muscles—from liver and brain by adsorption on aluminum hydroxide, elution with glycerophosphate buffer and precipitation by ammonium sulfate. These enzymes are not sufficient by themselves but require small amounts of adenylic acid as a coenzyme (11, 12, 13); on the other hand, Kiessling declares that adenylic acid is not necessary, inasmuch as enzyme preparations which have been freed of adenylic acid by dialysis are active. Cori interprets this apparent anomaly on the ground that the adenylic acid had not been removed quantitatively from Kiessling's preparation by the dialysis.

By repeated fractional precipitation with ammonium sulfate Kiessling obtained, both from rabbit muscle juice and from the juice resulting on maceration of yeast, proteins rich in phosphorylases and free from other enzymes; in particular, the extracts contained no phosphoglucomutase (see below).

Hanes (19) found that phosphorylases occur in many parts of plants. An especially satisfactory material is the extract of potatoes; the phosphorylase can be concentrated in it by fractional precipitation with ammonium sulfate. Green and Stumpf (16) succeeded in further enriching it to considerable extent.

Adenylic acid does not seem to be necessary as a coenzyme for this phosphorylase. All these phosphorylases attack starches as well as glycogen. In amylopectin, just as in glycogen, however, there are both  $\alpha$ -1,4 and  $\alpha$ -1,6 bonds to loosen, so that the question presents itself whether there are two different phosphorylases which are specific for 1,4 and 1,6 bonds, respectively. This can be decided by the action of the enzyme upon residual dextrin (obtained from amylopectin or glycogen

by decomposition with  $\beta$ -amylase), since it applies to the terminal groups which are represented in the formula on page 125. If the terminal groups which are linked in part by 1,6 bonds are broken off by phosphorylytic decomposition, then branches will be exposed to further attack by  $\beta$ -amylase. If the  $\alpha$ -1,6 bonds are not attacked, the dextrin will continue to resist  $\beta$ -amylase.

Phosphorylase from potatoes is unable to loosen 1,6 bonds, as K. H. Meyer and Bernfeld (33) found. This is a 1,4 phosphorylase. On the other hand, the phosphorylase from yeast can loosen both 1,4 and 1,6 bonds; undoubtedly it is a mixture of a 1,4 and a 1,6 phosphorylase.

Muscle certainly contains a 1,4 and a 1,6 phosphorylase. It will be shown later that both enzymes were present in the Kiessling preparation from muscle, whereas the purified preparation of Cori consisted only of 1,4 phosphorylase. The stability of residual dextrin toward 1,4 phosphorylase shows, above all, that the enzyme attacks the nonaldehydic end and not, for example, the middle. Its action is similar to that of  $\beta$ -amylase.

### 3. *The Phosphorolysis Equilibrium and the Enzymatic Synthesis of Polysaccharides*

That the reaction  $\text{Glycogen} + \text{Phosphate} \rightleftharpoons \text{Glucose-1-phosphate}$  is reversible and that from it one can obtain synthetic polysaccharides of the glycogen type was first shown by Schöffner and Specht (64) in a qualitative investigation. Directly afterward this reaction was studied more carefully by Kiessling (27) and by Cori and Hanes (19, 20). Kiessling obtained a suspension of the equilibrium:  $\text{Glycogen} + \text{Phosphate} \rightleftharpoons \text{Glucose-1-phosphate}$  with the two aforementioned protein fractions from muscle and yeast. Although three reactants are present in the equilibrium mixture, only the concentrations of the phosphate and of the glucose-1-phosphate enter into the equation:

$$\frac{\text{Inorganic phosphate}}{\text{Glucose-1-phosphate}} = K = 5.2$$

Cori's ester can also be converted in the absence of glycogen and phosphate to the extent of 84% (a more recent study by Cori (73) makes it 77%). Calorimetric determinations by Kiessling showed that the reaction is exothermic in the direction of glycogen formation, yielding 1100 calories per mol of glucose phosphate. Using potato phosphorylase, Hanes gives the constant for the reversible reaction as 6.

$$\frac{\text{Inorganic phosphate}}{\text{Glucose-1-phosphate}} = 6$$

From glucose-1-phosphate one can obtain a suspension of equilibrium and therewith the conversion of the bulk of glucose-1-phosphate into polysaccharides by addition of phosphorylase. The reaction has an autocatalytic character in this direction; it is catalyzed by polysaccharides, starch or glycogen (1,4). For the determination of enzyme activity it is necessary to suppress the period of induction by addition of a little starch or glycogen.

The polysaccharides obtained synthetically from glucose-1-phosphate have been isolated and investigated carefully. The one best characterized is the polysaccharide obtained by Hanes from potato extract by means of 1,4 phosphorylase. It exhibits all the properties of amylose; it is completely broken down by  $\beta$ -amylase (Hanes (20)), it shows the same x-ray diagram (Astbury, Bell and Hanes (2)) and it gives a pure blue color reaction with iodine. It consists, therefore, of an unbranched chain (amylose).

The polysaccharide obtained by Cori (4) from purified muscle phosphorylase has similar properties. It resembles amylose in the iodine reaction and the x-ray diagram. On the other hand, Kiessling obtained a product from his muscle enzyme having the properties of glycogen. We attribute the difference in the results obtained by both investigators to the circumstance that Cori employed pure 1,4 phosphorylase and Kiessling a mixture of 1,4 and 1,6 phosphorylase.

Cori (4) got a glycogen-like product from liver phosphorylase. The substance gave an amorphous x-ray diagram and a red-brown reaction with iodine.

#### 4. *The Breakdown of Glucose-1-Phosphate in the Liver*

In the breakdown of glycogen in the liver the reaction does not stop with glucose-1-phosphate; the latter is split up into glucose and phosphoric acid by a phosphatase which occurs in the liver. By addition of fluoride, the phosphatase can be poisoned. Then the autolysis of the glycogen stops with glucose-1-phosphate, even in unfractionated liver pulp (Ostern and Holmes (50)). Whether, along with the phosphorolysis, a direct hydrolysis of the glycogen in liver also takes place does not yet seem to be definitely decided.

#### 5. *The Breakdown of Glucose-1-Phosphate in Muscle and in Yeast; Glycogenolysis*

On undergoing decomposition in muscle the glucose-1-phosphate is rearranged into glucose-6-phosphate (Robison ester) by an enzyme, phos-

phoglucomutase, discovered by Cori (9, 11, 12). The reaction reaches equilibrium with 6% glucose-1-phosphate and 94% glucose-6-phosphate. The same rearrangement occurs also in yeast juice. Other reactions of glycogenolysis, *i. e.*, the transformation of glycogen into lactic acid (in muscle) and into alcohol (in yeast) will not be dealt with individually here. The following table is inserted for information:

#### THE REACTIONS OF GLYCOGENOLYSIS

(For a complete presentation see (47) and (25))

Glycogen + Phosphate 77%  $\rightleftharpoons$  Glucose-1-phosphate 23%

Glucose-1-phosphate 6%  $\rightleftharpoons$  Glucose-6-phosphate 94%

Glucose-6-phosphate 85%  $\rightleftharpoons$  Fructose-6-phosphate 15% (28)

Fructose-6-phosphate + Adenosin triphosphate  $\rightarrow$  Fructose-1-6-diphosphate + Adenosin diphosphate (51)

Fructose-1-6-diphosphate  $\rightleftharpoons$  Glyceric aldehyde-3-phosphate + dioxycetone-1-phosphate

Dioxyacetone-1-phosphate  $\rightleftharpoons$  Glyceric aldehyde-3-phosphate (42)

Glyceric aldehyde-1-phosphate + Phosphate  $\rightleftharpoons$  Glyceric aldehyde-1-3-diphosphate (43)

Glyceric aldehyde-1-3-diphosphate + Codehydrase  $\rightarrow$  Glyceric acid-1-3-diphosphate + Adenosin triphosphate (44)

Glyceric acid-3-phosphate  $\rightleftharpoons$  Glyceric acid-2-phosphate (29)

Glyceric acid-2-phosphate  $\rightleftharpoons$  Pyruvic acid-2(enol) phosphate (54)

Pyruvic acid-2-phosphate + Adenosin diphosphate  $\rightarrow$  Pyruvic acid + Adenosin triphosphate (3)

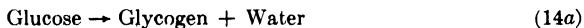
(a) Muscle: Pyruvic acid + Dihydrocodehydrase  $\rightarrow$  Lactic acid + Codehydrase

(b) Yeast: Pyruvic acid  $\rightarrow$  Acetaldehyde + CO<sub>2</sub>

Acetaldehyde + Dihydrocozymase  $\rightarrow$  Alcohol + Cozymase

#### 6. Biological Synthesis of Glycogen

The storage of glucose as glycogen, *i. e.*, the reaction:



cannot proceed without additional supply of free energy. That is to say, it requires coupling with a second energy-supplying reaction, since the equilibrium in presence of water favors the glucose formation almost exclusively. This suggests the assumption that the progenitor of glycogen formation is glucose-1-phosphate which can yield glycogen in a reaction proceeding spontaneously. There has been no success as yet, however, in attempts to obtain glucose-1-phosphate directly by an enzymatic reaction from glucose and one of the phosphorus donors which form in the course of sugar decomposition, for the phosphorus donors, such as diphosphoglyceric acid, phosphorylate glucose in the 6 position. However, the glucose-6-phosphate can rearrange itself into glucose-1-phosphate to a



certain percentage under the influence of phosphoglucomutase (see p. 130). If this is transformed into glycogen and the equilibrium is thereby destroyed, additional 6,phosphate can rearrange into 1,phosphate. The energy-supplying reaction with which the storing up of glucose is coupled may, therefore, be the dehydrogenation of diphosphoglyceric aldehyde to give diphosphoglyceric acid; part of the sugar is broken down by oxidation so as to supply the energy for storing up the preponderance of the sugar.

## IX. The State of Glycogen in the Organs

### 1. Glycogen and Albumin

Glycogen reacts in some way with many dissolved proteins in aqueous solution, for an extract of organs which contains glycogen and has not been freed of albumin is not broken down either by the  $\alpha$ -amylase of *Aspergillus* (takadiastase) (Tsai (75)) or by  $\beta$ -amylase (K. H. Meyer and Press (41)). Only when the albumin has been precipitated, *e. g.*, by tungstic acid, is the glycogen attacked.

If glycogen or starch is added to a weakly alkaline solution of myosin, the opalescence is intensified—a sign that the particles have become coarser. Then, on neutralization, the precipitated protein carries down a considerable amount of carbohydrate with it. Przylecki (57) believes that myosin and starch form a compound of fairly constant composition containing 20% starch. On the other hand, no constant value is obtained in the case of glycogen.

### 2. The State of Glycogen in the Organs

The question whether glycogen in the native state is combined with albumin has often been debated. In our opinion the evidence discussed in the foregoing is not sufficient to settle the matter. According to the results reported on page 126, a union by primary valences can be regarded as out of the question. On the other hand, the glycogen stored in the organ and the surrounding albumin may turn out to exert some kind of valence force upon each other. At best, therefore, one can only consider whether or not Przylecki may speak of a specific combination—a symplex. It has already been shown in earlier papers that by a single extraction of muscle or liver pulp with hot water only a part of the glycogen goes into solution. Pflüger attributed this to a partial surrounding of the glycogen by coagulated albumin.

Willstätter and Rohdewald (76) are of the opinion that the insolubility

of part of the glycogen is due to a combination with protein. They call the bound glycogen *desmogleycon* and the unbound, extractable glycogen *lyoglycon*. They find that the proportion of glycogen in the lyo- form depends upon how well fed the animal is. The richer in glycogen the greater the proportion of lyoglycon. Goose livers with 4 to 8% glycogen contained 90 to 99% of it as lyoglycon. Livers which were low in glycogen, containing 0.53 to 1.25%, had only 12 to 50% of it as lyo- and 88 to 50% as desmogleycon. The distinction between the "lyo" and "desmo" forms is, moreover, somewhat arbitrary. For example, the manner of extracting plays an important role.

It is our opinion that in evaluating these investigations it is especially important to keep in mind the fact that glycogen itself occurs in different degrees of polymerization with different solubilities. The "desmogleycon" seems to us to be nothing else than the high-polymeric fraction of glycogen which is insoluble in water and soluble in alkali. From the Willstätter-Rohdewald research it would then follow that organs rich in glycogen contain a large proportion of the lower-polymeric glycogen. With regard to the question of how the glycogen is bound, we hold the same opinion as that expressed by Pflüger in his book in 1901, namely, that there are no grounds for assuming a union between glycogen and albumin in the organs, as the variable solubility of the glycogen is enough in itself to explain the phenomena but that the possibility of such a union cannot be excluded with certainty.

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I am deeply indebted to Miss M. Fuld and to Mr. R. Jeanloz for their very helpful collaboration.

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# VERDOPEROXIDASE

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## I. Introduction

Verdoperoxidase (V.P.O.) is a ferment that has been isolated from leucocytes. It is green in color and catalyzes peroxidatic reactions.

The occurrence in leucocytes of a substance that catalyzes what have sometimes been supposed to be peroxidatic, and sometimes oxidatic, reactions, has been known ever since Klebs in 1868 (1) and Struve in 1872 (2) showed that guaiac tincture takes on a blue color in the presence of pus. In 1899 Achalmé (3) observed that the formation of indophenol blue from a mixture of  $\alpha$ -naphthol and diethyl-paraphenylenediamine (the Nadi reagent) was hastened.

The effect of the active substance was shown, not only in pus, but also in leucocyte-rich tissues by Brandenburg in 1900 (4) and Meyer in 1903 (5). Bone marrow and blood from patients with myeloid leucemia, in contradistinction to organs such as lymph glands, thymus and spleen, showed a positive reaction. By the extraction of pus both these authors obtained active preparations, and Meyer demonstrated that the activity accompanied the fractions that were precipitable with alcohol and ammonium sulfate. Despite these procedures, Meyer did not observe the characteristically colored verdoperoxidase that almost certainly must have existed in his preparations. As will be shown

later, V.P.O. in pus and similar material is bound to substances that "conceal" the verdoperoxidase color—substances that he was unable to remove.

In 1907 Winkler (6) showed that on staining with Nadi reagent small blue grains were deposited in the cytoplasm in myeloid cells, whereas other cells, such as lymphocytes, for example, were not stained. This staining, like certain others, such as the method of staining with benzidine and hydrogen peroxide described by Fischel in 1910 (7), has been of great importance in morphological studies of white blood corpuscles for ascertaining whether existing cells are to be classified as myeloid forms ("oxidase-positive") or not.

Whether the activating substance in leucocytes is to be regarded as an oxidase or as a peroxidase has not been definitely shown. It has, as a rule, been given as an oxidase. As early as 1898, however, Linossier (8) showed that a staining reaction with *guaiaci resina* and similar chromogens in the presence of pus took place only when the reagent contained hydrogen peroxide or else on addition of the same. The reaction was accordingly peroxidatic. When staining leucocytes with Nadi reagent hydrogen peroxide is not added. Hydrogen peroxide is, however, formed in the reagent by autoxidation. An addition of catalase to the reagent before the staining prevents the formation of indophenol blue (Agner, 1941 (9)). Thus, this reaction also is to be regarded as peroxidatic, and the active substance should be described as a peroxidase.

The isolation of this ferment and a description of certain of its properties have recently been given (Agner, 1941 (9)). The substance obtained has been isolated from empyemic fluid, from leucocytes taken from a patient with myeloid leucemia, and has also been shown in chloro-leucemic infiltrates. It is green in color and has a peroxidase effect. Because of its characteristic color it has been named verdoperoxidase to distinguish it from other peroxidases.

## II. The Preparation of Verdoperoxidase

The material for the preparation of V.P.O. has consisted of empyemic fluid from tuberculous patients. The empyemic fluid has a greenish gray appearance and examination with the spectroscope shows absorption bands at 625 and 580  $m\mu$ ; after reduction one observes two sharp absorption bands at 637 and 559  $m\mu$ . The empyemic fluid has a strong peroxidase effect. In a substance like empyemic fluid one finds the substances that cause the typical absorption bands and the peroxidase effect both in the cells and in the fluid surrounding these. The verdoperoxidase is bound to the leucocytes and is probably released on the disintegration of the structures; it can then be demonstrated also in the fluid surrounding the cells. In cell-free pleural exudate, on the other hand, no absorption bands can be shown.

That V.P.O. is not merely a conversion product of hemoglobin or similar

substance is proved by the fact that it has been possible to prepare the ferment from leucocytes in a case of myeloic leucemia.

The method of preparation that has so far given the best result is described below.

*First Step: Preparation in a Layer Between Ether and Ammonium Sulfate Solution*

To one volume of empyemic fluid add two volumes of distilled water and one volume of ether. Shake the mixture. Add 400 gm. of ammonium sulfate per 1000 ml. of mixture and centrifuge the solution. A yellow-colored ether layer is obtained on the top, and under this a relatively solid "cake," while at the bottom there is a clear, colorless solution. Suck off the ether and water phases and dissolve the middle layer in water.

The resulting solution is very viscous and has a greenish gray appearance.

*Second Step: Purification with Barium Acetate*

Add to the above-mentioned solution an amount of saturated barium acetate solution determined by preliminary experiments, upon which large, white, heavy flakes are precipitated. The suitable amount of barium acetate must be ascertained by a preliminary test in every preparation. Too much barium acetate must not be added, or the solution will become opalescent after centrifugation.

After this precipitation with barium the solution is quite clear, and has a relatively strong, beautiful green coloring. It may seem somewhat strange to add barium salts to a solution containing sulfate. In this case, however, it has proved very practical. The substances that "conceal" the color of V.P.O. and give rise to the high viscosity in the solution are removed. This precipitation facilitates the subsequent steps in the work of preparation.

*Third Step: Precipitation with Alcohol*

Add to the solution an equal volume of 95% ethyl alcohol. After strong centrifugation a white deposit (3 A) is obtained. Add to the supernatant yellow green liquid a further amount of ethyl alcohol so that the concentration of ethyl alcohol becomes 65 vol. %. Dissolve the strongly green-colored precipitate (3 B) in water. The mother liquid (3 C), which is red in color, is evaporated and dialyzed at a low temperature.

*Fourth Step: Ammonium Sulfate Precipitation*

Add an equal volume of saturated ammonium sulfate solution to the solution (3 B). An almost colorless precipitate (4 A) is formed. Centrifuge. By adding a further amount of ammonium sulfate to make the degree of saturation equal 0.65, one obtains



a green precipitate (4 B). The ammonium sulfate concentration is increased in the mother liquid to 80–90%, upon which a reddish brown precipitate (4 C) appears.

#### *Fifth Step: Ammonium Sulfate-Barium Acetate Treatment*

The green-colored precipitate (4 B) is dissolved in water and barium acetate is added. Repeated precipitations of V.P.O. with ammonium sulfate accompanied by additions of barium acetate are carried out. This treatment results in the removal of substances that are bound to V.P.O. The V.P.O. preparation is dialyzed against distilled water. If in this connection a precipitate containing V.P.O. appears, it is centrifuged from the soluble part of the preparation. The precipitated fraction is dissolved in 1% sodium chloride solution and subjected to renewed ammonium sulfate-barium acetate treatments.

#### *Sixth Step: Electrophoresis*

The part of the ferment that is soluble in distilled water is subjected to electrophoresis in phosphate buffers, pH 6.8, ionic strength 0.1, 0° C. The fraction that moves to the positive pole with an electrophoretic mobility of  $2.0 \times 10^{-8}$  cm.<sup>2</sup>  $\times$  volt<sup>-1</sup>  $\times$  sec.<sup>-1</sup> is isolated.

The reduced form of V.P.O. has a strong absorption band at 637 m $\mu$  (page 141). The yield of ferment in connection with the preparations is determined by absorption measurements at this wave length both before and after reduction. The difference between the absorption coefficients for the oxidized and reduced forms of the preparation in the purest state amounts to a value of  $2.45 \times 10^7$  cm.<sup>2</sup> per gram-atom of ferment-bound iron in 1 ml. of solution.

The following is a survey of the method for the preparation together with data concerning the yield in connection with a preparation carried out by the author.

1. 850 ml. of empyemic fluid + 1500 ml. of distilled water + 850 ml. of ether + 1200 gm. of ammonium sulfate. The preparation contained  $7.2 \times 10^{-6}$  gram-atoms of ferment-bound iron, corresponding to about 400 mg. of pure V.P.O.

2. Addition of barium acetate:  $6.3 \times 10^{-6}$  gram-atoms of ferment iron. Yield, 88%.

3. Precipitation with ethyl alcohol:

A. 45%: colorless precipitate;

B. 65%: green precipitate.  $4.4 \times 10^{-6}$  gram-atoms of ferment iron. Yield, 70%.

C. Red mother liquid, which is evaporated to small volume.

4. The precipitate 3 B is dissolved in water and precipitated with ammonium sulfate:

A. 50% saturation: colorless precipitate;

B. 65% saturation: green precipitate.  $3.7 \times 10^{-6}$  gram-atoms of ferment iron. Yield, 84%.

C. 80–90% saturation: red precipitate.

5. Precipitate 4 B is dissolved in water and treated with ammonium sulfate and barium acetate until a preparation soluble in distilled water is obtained.

6. Electrophoresis. Phosphate buffer: pH 6.8; ionic strength 0.1; temp. 0° C. The fraction with positive charge consists of a green substance = V.P.O.

The total quantity of material purified in this way was 140 mg., corresponding to  $2.5 \times 10^{-6}$  gram-atoms of ferment iron; 35% of the original amount was obtained in yield.

Besides V.P.O. two other colored substances were isolated in the course of the preparation, one red substance in solution (3 C) and a reddish brown precipitate (4 C). These substances have not yet been investigated in detail.

The first-mentioned substance has absorption bands at 625 and 538  $m\mu$ , after reduction a very sharp  $\alpha$ -band at 559 and a less sharply defined  $\beta$ -band at 529  $m\mu$ . These characteristics are distinguishing features of parahematin and hemochromogens, and the ferment is probably to be classified under the group of the b-cytochromes. The other substance is brownish red in color, and has an absorption band at 495  $m\mu$ . Its properties and possible functions have not been investigated.

### III. Spectroscopic Investigations and Light Absorption

Verdoperoxidase in solution has in the oxidized form a brownish green color and shows absorption bands in the visible part of the spectrum at 690, 625, 570 and (500)  $m\mu$ , as well as end absorption from 465  $m\mu$ . On reduction the color turns bright green and absorption bands are observed at 637 and 475  $m\mu$ .

V.P.O. forms a peroxide compound with hydrogen peroxide. The color of this peroxide compound resembles that of reduced V.P.O. absorption band at 625  $m\mu$ . This compound is instable and gradually disintegrates, with liberation of the V.P.O. in the oxidized form. The peroxide compound oxidizes certain substances, *e. g.*, hydroquinone, when the V.P.O. itself changes to the normal ferri-form.

With hydrogen cyanide, hydroxylamine and azide, V.P.O. forms compounds, and these show the characteristic absorption bands given in the table below. It may here be mentioned that it is the oxidized form that reacts with hydrogen cyanide and hydroxylamine, while azide seems to form a compound with reduced V.P.O.

Carbon monoxide and fluoride do not give spectroscopically observable compounds.

#### SURVEY OF THE SPECTROSCOPIC RESULTS

V.P.O. <sub>ox.</sub> .....		690, 625, 570 (500) $m\mu$
V.P.O. <sub>red.</sub> .....		637, 475 $m\mu$
V.P.O. <sub>ox.</sub> .....	+H <sub>2</sub> O <sub>2</sub>	625 $m\mu$
V.P.O. <sub>ox.</sub> .....	+HCN	634, 458 $m\mu$
V.P.O. <sub>ox.</sub> .....	+H <sub>2</sub> NOH	628, 460 $m\mu$
V.P.O. <sub>red.</sub> .....	+NaN <sub>3</sub>	615, 460 $m\mu$
V.P.O. <sub>ox.,red.</sub> .....	+NaF	As V.P.O. <sub>ox.,red.</sub>
V.P.O. <sub>ox.,red.</sub> .....	+CO	As V.P.O. <sub>ox.,red.</sub>

Figure 1 shows spectra for oxidized and reduced V.P.O. measured with a photoelectric apparatus essentially according to Warburg and Negelein (10).

Absorption bands at 430  $m\mu$  for the oxidized and at 475  $m\mu$  for the reduced form are characteristic for porphyrins and are found in the absorption curves for all examined substances containing iron-porphyrin groups. The absorption coefficient  $16.1 \times 10^7$   $\text{cm}^2/\text{gram-atom}$  of iron is

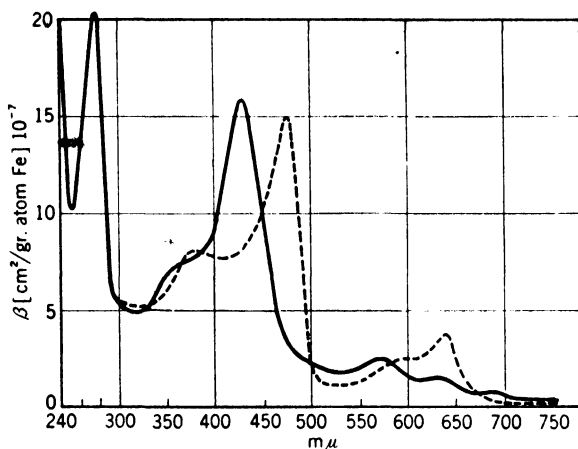


Fig. 1.—Spectra of oxidized and reduced verdoperoxidase preparation (Agner, 1941).

The absorption coefficient was calculated from the formula

$$\beta = \ln \frac{I_0}{I} \cdot \frac{1}{c} \cdot \frac{1}{d} \text{ cm.}^2/\text{gr.-atom of iron}$$

$I_0$  represents the intensity of the incident light and  $I$  the intensity of the transmitted light.

$d$  = the layer thickness in cm.

$c$  = gram-atoms Fe/ml.

————, oxidized V.P.O.

-----, reduced V.P.O.

of the same order of magnitude as for the Soret's band of other hemin catalysts. This circumstance constitutes one of the supporting facts for the assumption that the prosthetic group in V.P.O. is an iron-porphyrin compound.

#### IV. Analysis of Verdoperoxidase

The preparation of V.P.O. purified in the above-described manner proved on cataphoresis at pH 6.8, 8.5 and 10.6 to be completely homo-

geneous, which favors the assumption that the preparation is in a pure or almost pure state.

The nitrogen content for this V.P.O. preparation was 17.15% and the iron content 0.1%, which value corresponds to  $1.8 \times 10^{-5}$  gram-atoms per gram of preparation. Besides iron, the preparation contained 0.001% of copper.

The concentration of the group that on reduction changed the color of the V.P.O. and gave rise to the changes in position and size of the absorption bands, was determined by anaerobic titration with hydrosulfite solution. Hydrosulfite solution was added in determined quantities to a solution of V.P.O. in the oxidized state and the change in the absorption at 637 m $\mu$  was measured with the aid of the photoelectric apparatus. The change was in an even proportion to the addition of hydrosulfite up to the amount at which all the V.P.O. had been reduced. The hydrosulfite added was compared with an amount that was used up for the reduction of a definite quantity of ferri-salt. From this result was calculated the amount expressed in gram-atoms that had been reduced. The value was  $1.97 \times 10^{-5}$  gram-atoms per gram of dry substance. This value is in close conformity with the value for the iron content  $1.8 \times 10^{-5}$ , which makes it probable that iron is contained in the color-conditioning group.

The absorption measurements have shown that porphyrin probably enters into the prosthetic group; and in the light of the analyses mentioned above it is likely that it consists of iron-porphyrin compounds.

Hemoglobin and protohemin catalyzers, *e. g.*, catalase and peroxidase, are split by acetone containing hydrochloric acid into protein component and hemin. A similar splitting off of iron-porphyrin groups from V.P.O. is not possible. Nor has the prosthetic group yet been isolated in any other way. After investigations by Haurowitz (11) sulfhemoglobin has been described as a green-colored substance which in contradistinction to hemoglobin, cannot be split with acetone-hydrochloric acid into protein and prosthetic groups. V.P.O. has thus in this respect certain properties that remind one of sulfhemoglobin. It is also impossible to split cytochrome c with acetone-HCl, which in this case is due to additive bindings of cystein-SH to the vinyl groups (Theorell (12)).

## V. The Activity of Verdoperoxidase

The isolated green-colored substance has been examined as to its capacity to act as an oxidase, catalase and peroxidase, respectively. Hydroquinone, paraphenylenediamine and ascorbic acid are not oxidized in the

absence of hydrogen peroxide; on addition of the same, however, a relatively rapid oxidation took place. The catalase effect of the ferment was extremely slight.

Like peroxidase from horseradish (Theorell and Swëdin (13a, b)) V.P.O. acts as a dihydroxymaleic acid oxidase. Theorell's explanation of this is that dihydroxymaleic acid oxidase is not to be considered as an oxidase in the strict sense, and that the oxidation of dihydroxymaleic acid is to be regarded as a reaction in which hydrogen peroxide is formed in

intermediate reactions. In this connection peroxidase has a catalyzing effect.

Substances that are easily oxidized in the reaction catalyzed by the ferment are catechol, pyrogallol, hydroquinone, paraphenylenediamine and ascorbic acid. Less easily oxidized are resorcinol and benzidine, while phenol, tyrosin and ethyl alcohol are not oxidized.

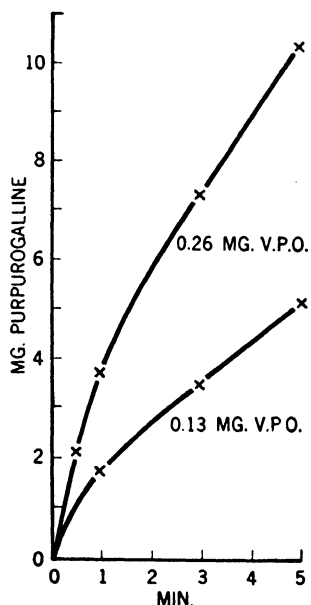


Fig. 2.

For quantitative determinations of the activity of peroxidase preparations the colorimetric method is generally used, where the amount of purpurogallin formed from pyrogallol is determined. Under the standard conditions for this method described by Keilin and Mann (14) V.P.O. showed a purpurogallin number = 41. For the period of five minutes during which the determination is to be carried out, however, V.P.O. is inactivated in the manner shown in Fig. 2. The color development for varying amounts of preparation is determined

after different times. If the calculation for the purpurogallin number is performed with the aid of the values for the initial rate, it will amount to about 75.

As early as 1907 v. Czylartz and v. Fürth (15) observed that the peroxidase effect of leucocytes had just such a course as that described above, the color development during the first periods (they used leuco-malachite green and hydrogen peroxide as substrate) being strongest and afterward diminishing. Further, they showed that the peroxidase effect for hemoglobin and other hemin compounds, on the other hand, was directly proportional to the time. This difference in effect was taken by the authors as

a proof that the peroxidase effect caused by leucocytes was conditioned by a special ferment, and was not an unspecific effect of hemin derivatives occurring in the preparation. The question as to whether there was any peroxidase at all in leucocytes had been raised in connection with Moitesier's demonstration (1904 (16)) of the capacity of hemin compounds to catalyze peroxidatic reactions. All such reactions were at that time thought to be due to an unspecific hemin effect, and this view still occurs in the literature. That hemin compounds have a peroxidatic effect has been definitely proved; but this effect is very weak in relation to that of the peroxidatic ferments.

The activity of V.P.O. is scarcely  $1/10$  of that of horseradish peroxidase. However, the V.P.O. content of the leucocytes is so great that the peroxidase effect in leucocytes is considerable. The leucocytes in a case of myeloid leukemia were isolated. V.P.O. was extracted and prepared, and the ferment content was estimated at about 1-2% of the dry weight of the leucocytes. In comparison with the peroxidase effect in horseradish root, the part of the plant that is stated to be one of the most rich in peroxidase, the effect for leucocytes is calculated to be about six times as great.

V.P.O. is relatively stable when subjected to heat. At 80° C. the ferment is inactivated in 10 minutes only by 6.4%. At 90° C., however, the inactivation is complete after 10 minutes.

V.P.O. is inactivated by  $N/1$  sodium hydroxide and by  $N/10$  hydrochloric acid. No inactivation, on the other hand, is brought about in  $N/10$  sodium hydroxide and  $N/10$  acetic acid.

V.P.O. is inhibited by hydrogen cyanide, hydroxylamine, azide and fluoride; the ferment is not inhibited by carbon monoxide.

The ferment is inactivated only by about 10% if it is precipitated with a mixture of ethyl alcohol and formalin in which the respective concentrations are 70 and 10%. As regards the "oxidase coloring" of leucocytes, this is generally performed in alkaline solution of  $\alpha$ -naphthol and diethyl-paraphenylenediamine after fixing the preparation in alcohol + formalin. As a rule, of course, one cannot expect ferments to stand up to such a drastic treatment, and various attempts were formerly made to explain how the oxidase reaction can remain after fixation. It has been assumed that the activating substance was of a non-enzymic nature, or that ferments were protected by certain substances. V.P.O. is, however, resistant to treatment with both alkali  $N/10$  and ethyl alcohol + formalin; and we find here the explanation of the "stainability" of the leucocytes under the above-mentioned conditions.

## VI. Discussion

Peroxidase preparations produced from different starting materials have proved to differ both as regards properties and as regards the manner of reacting.

The commonest material for preparation has been horseradish root. Preparations from this material have proved to contain two different peroxidases: peroxidase I and peroxidase II (17), now called "paraperoxidase" and "peroxidase," respectively (18). Peroxidase has been crystallized by Theorell in 1941 (18, 19).

Altschul, Abrams and Hogness (20) have prepared peroxidase from yeast. This ferment has an appearance and a spectrum that remind one of horseradish root peroxidase. Its effect, however, is specifically adapted for an oxidation of reduced cytochrome c. It was first described as an oxidase, and not until it had been more closely investigated did it appear that it was active only in the presence of hydrogen peroxide.

It has long been known that milk has a strong peroxidase effect. Peroxidase from this source has been prepared in connection with a number of investigations, though not in a form in which it was possible to observe it more closely. It is brownish in color.

Verdoperoxidase has a color and a spectrum that diverge from those of earlier described peroxidase preparations. On examination it has proved to be active with ordinary peroxidase reagents. Quantitative determination of the effect in connection with the formation of purpurogallin showed the same to be only  $1/10$ – $1/20$  of that shown by plant peroxidase. The function of V.P.O. in the leucocytes has as yet not been investigated in detail. V.P.O. occurs in high concentration in the leucocytes. It would seem strange if this high content of V.P.O. were needed merely for the oxidative conversion in the leucocytes. It is conceivable that it also has a function in connection with the general reaction of the leucocytes to infection and similar states.

Leucocytes give rise to the formation of indophenol blue with the Nadi reagent. The activating substance has hitherto been described as an oxidase. Hydrogen peroxide, however, is formed in the reagent by autooxidation, and the color formation is catalyzed by V.P.O.

The formation of indophenol blue that is caused by tissues is inhibited or in some cases topped altogether on addition of catalase. One should thus ask oneself whether peroxidases do not, here also, play a role, as Vernon (21) assumed as early as 1912. Perhaps the occurrence of peroxidases is much more general than we have hitherto had occasion to suppose.

The isolated peroxidases are colored substances, and like the cytochromes whose presence has been demonstrated in the tissues they have typical absorption bands. These absorption bands of the cytochromes have shown a certain variation on spectroscopic observation, and the conception of especially the  $\alpha$ -cytochromes has therefore varied greatly. In several species of bacteria and tissues the typical  $\alpha$ -band at  $605\text{ m}\mu$  is missing, and, instead, bands around  $590$  and  $630\text{ m}\mu$  are found. These typical  $\alpha$ -cytochrome bands have been designated  $\alpha_1$  and  $\alpha_2$ , respectively. Warburg and others (22) have asserted that in any case certain of the observed substances are ferments with specific functions. Keilin (23) and others, on the other hand, consider these substances to be more or less modified cytochrome- $\alpha$  derivatives. According to Keilin, if only its absorption bands in the leucocytes had been observed and its effect not been ascertained in detail, V.P.O. would have probably been described as belonging to the cytochrome- $\alpha_2$  group.

The  $\alpha_2$ -cytochromes with absorption bands in the vicinity of  $630\text{ m}\mu$  have been regarded by Lemberg and Wyndham (24) as bile pigment hemochromogens (verдохemochromogens). V.P.O.'s absorption band at  $637\text{ m}\mu$  would thus lead to the assumption that the prosthetic group was of a similar nature. Certainly, the constitution of V.P.O. has not been investigated in detail; but it may nevertheless be stated that it has not the properties that are considered characteristic for bile pigment hemochromogens.

In the author's opinion there is but little justification for drawing conclusions about the properties or constitution of a substance simply on the basis of spectroscopic similarities or dissimilarities. Substances differing greatly in color and absorption have shown similar effects, *e. g.*, plant peroxidase and V.P.O. Plant peroxidase and cytochrome- $c$  peroxidase have similarities as to color and absorption, but differ in their way of taking effect. Among the substances that have been classified under the cytochromes there are perhaps some with effect other than the reversible reaction  $\text{ferric} \rightleftharpoons \text{ferrous}$  ascribed to the cytochromes.

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# MECHANISMS OF CARBOHYDRATE METABOLISM. AN ESSAY ON COMPARATIVE BIOCHEMISTRY

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*"Every system which has not reached a state of equilibrium is changing continuously towards such a state with greater or less speed."*

—LEWIS and RANDALL\*

## I. Introduction

It is a general belief that, when living cells made their first appearance in this world, they started using the reduction of  $\text{CO}_2$  as a source of energy, these unicellular organisms being able to live with the sole aid of inorganic

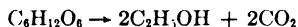
The following abbreviations have been used: ATP = adenosinetriphosphate; ADP = adenosinediphosphate; Ad = adenylic acid; Cr = creatine; Arg = arginine;  $\text{Pyr}(\text{PO}_4)_2$  = diphosphopyridine nucleotide;  $\text{Pyr}(\text{PO}_4)_3$  = triphosphopyridine nucleotide; DPT = diphosphothiamine.

\* Quoted from *Thermodynamics*, 1923, p. 18.

substances and  $\text{CO}_2$  ( $4\text{H}_2\text{A} + 2\text{CO}_2 \rightarrow 4\text{A} + \text{CH}_4 + 2\text{H}_2\text{O}$ ;  $4\text{H} + \text{CO}_2 \rightarrow (\text{CH}_2\text{O}) + \text{H}_2\text{O}$ ;  $\text{CO}_2 + \text{H}_2 \rightleftharpoons \text{HCOOH}$ ;  $2\text{CO}_2 + 2\text{H}_2 = \text{CH}_3\text{COOH}$ ). With the formation of these carbon-containing building stones, another reaction between some of these substances and  $\text{CO}_2$  became possible, fixation of  $\text{CO}_2$  ( $\text{RH} + \text{CO}_2 \rightleftharpoons \text{RCOOH}$ ); these series of dark, exergonic, wasteful reactions were complemented by another series of more efficient, endergonic reactions when chlorophyll-protein was formed, acting as the mediator for the series of reactions:



Once the carbohydrate molecule was formed, it became the main source of energy for the living cell ( $\Delta F^\circ_{298}$  of glucose  $\rightarrow 6\text{CO}_2 + 6\text{H}_2\text{O} = -217,020$  cal. (165)). Lack of electron mediators toward oxygen prevented at first the full utilization of the free energy released on the complete combustion of carbohydrate, and the anaerobic breakdown, fermentation, must have been the primitive method of carbohydrate breakdown. Fermentation ended with the formation of alcohol and  $\text{CO}_2$ , the Gay-Lussac equation:



Plants, yeasts, molds, and some bacteria still have alcoholic fermentation. With the introduction of Fe into the etioporphyrin molecule (porphyrine nucleus of chlorophyll) the cells came into possession of catalysts for the oxidation of carbohydrates by atmospheric oxygen, and there was another end product in fermentation, lactic acid: ( $\text{C}_6\text{H}_{12}\text{O}_6 \rightarrow 2\text{C}_3\text{H}_6\text{O}_3$ ). The fermentation process became more and more efficient by the introduction of a series of reversible steps made possible by the introduction of phosphorus. The oxidative process, too, added in its way toward oxygen more and more reversible steps. It was logical that these two processes with so many reversible links should be connected to one another through one or more of these links; this relation the genius of Pasteur saw in all its complexity. Unicellular organisms became multicellular organisms, and the creation of a new phase—the intercellular phase—brought forth new complexities in the production of energetic processes; and secretory processes and excretory processes contributed to maintain the “fixité du milieu intérieur.” As the activities of the multicellular organisms increased, the utilization of fuel became more and more efficient, efficiency obtained by the introduction of energy-rich phosphate bonds. As the evolutionary process advanced, control of energy production appeared, the rate of the manifold enzymatic reactions becoming controlled by a number of chemical factors, which in the vertebrate organisms reached marked specificity and constituted the hormonal mechanism of control. In this gradual evolution of living organisms it is reasonable to assume that the intricate machinery of fermentation enzymes and oxidation enzymes, all controlled by the hormonal mechanism of regulation, must have evolved through a long process of seemingly gradual complications but actually all tending to attainment of complete reversibility. Have the different evolutionary steps disappeared, and is the mechanism of fermentation and oxidation enzyme systems thus uniform throughout living cells? Are there still left remnants of those wasteful processes of carbohydrate metabolism? Comparative biochemistry, to provide an answer to these

questions, must devote special attention to the mechanism of carbohydrate metabolism in plants and protozoa, and their regulating mechanisms. Furthermore, on studying these mechanisms, special attention must be paid to the differences observed among living cells.

Comparative biochemistry must also be always on the alert for hasty applications and hasty generalizations of work with isolated systems, not only because the rate of reaction and the orientation of reaction are controlled by nonenzymatic mechanisms, but also because some of those isolated systems become reactive only after destruction of the architecture of the living cell and the disappearance of the controlling mechanisms. An attempt has been made in this article to study the metabolism of carbohydrates in living cells with the guidance of the considerations stated above. The meagerness of necessary data has been a great obstacle in the realization of this attempt. It is hoped that the gaps that have been found may be filled as soon as conditions for investigative work become normal again.

## II. Catabolic Process: Carbohydrate Fermentation

### 1. First Phase: *Glycogen* $\rightarrow$ *Pyruvate*

Fermentation, the anaerobic phase of carbohydrate breakdown, may end in the formation of either lactic acid or alcohol. In any event, both end products have a common pathway down to the formation of pyruvate; moreover, under aerobic conditions the fermentation or anaerobic phase may stop in the pyruvate stage, which then may undergo the series of oxidative reactions. Pyruvate is the *obligatory* end product of fermentation in the normal pathway of carbohydrate metabolism.

The clarification of the mechanism of this first phase started with the discovery by the Russian botanist Ivanow (90) of the formation of phosphorylated compounds in plant fermentation, and ended with the discovery by Warburg of the mechanism of the linkage between phosphorylation and oxidation-reduction (212). Without the outstanding discoveries made by the laboratories of Meyerhof, Embden, Parnas, and Cori, who unraveled the series of reactions, the last achievement would have been impossible. Let us consider first the phosphorylated formation of pyruvate. In the breakdown of the six-carbon molecule, glucose, into the three-carbon molecule, pyruvate, there are eight separate steps, all of them reversible but No. 7 (Table I), the dephosphorylation of phosphopyruvic acid by adenylic acid to pyruvate. If the process starts from a polysaccharide—glycogen or starch—three more reversible systems are added. Whether the process starts from glycogen or from glucose, the phosphate molecule present in all the intermediate states is absent in the initial and final states, showing the catalytic character of these phosphorylations.

This series of reactions, in which phosphate is bound as ester, transferred in intramolecular migrations, or converted into energy-rich phosphate

bonds, occurs in a variety of ways. Thus, in reaction 1 (glycogen +  $\text{H}_3\text{PO}_4 \rightleftharpoons$  glucose 1-phosphate) and in reaction 8 (3-glyceraldehyde phosphate +  $\text{H}_3\text{PO}_4 \rightleftharpoons$  1,3-diphosphoglyceraldehyde) there is introduction of inorganic phosphate; while the first, however, leads to the formation of an energy-

TABLE I  
CARBOHYDRATE FERMENTATION BY PHOSPHORYLATION.  
FIRST PHASE: CARBOHYDRATE  $\rightarrow$  PYRUVATE

No.	Enzyme system	Reacting systems	References
1	Phosphorylase (protein-Mg-adenylic acid)	Glycogen + $\text{H}_3\text{PO}_4 \rightleftharpoons$ glucose 1-phosphate	(47)
2	Phosphoglucosyltransferase (protein-Mg)	Glucose-1-phosphate $\rightleftharpoons$ glucose 6-phosphate	(48, 193)
3	Isomerase (hexose) (protein-M*)	Glucose 6-phosphate $\rightleftharpoons$ fructose 6-phosphate	(123)
4	Phosphohexokinase (protein-ATP ?)	Fructose 6-phosphate + ATP $\rightleftharpoons$ fructose 1,6-phosphate + ADP	(163)
5	Hexokinase (protein-ATP ?)	Glucose + ATP $\rightleftharpoons$ glucose 6-phosphate + ADP	(132)
6	Zymohexase (protein-M*)	Fructose 1,6-phosphate $\rightleftharpoons$ dioxyacetone phosphate Fructose 1,6-phosphate $\rightleftharpoons$ 3-glyceraldehyde phosphate	(83, 136, 137)
7	Isomerase (triose) (protein-M*)	Dioxyacetone phosphate $\rightleftharpoons$ 3-glyceraldehyde phosphate	(138)
8	Nonenzymatic	3-Glyceraldehyde phosphate + $\text{H}_3\text{PO}_4 \rightleftharpoons$ 1,3-diphosphoglyceraldehyde	(151, 211)
9	Phosphoglyceraldehyde oxidase (protein-DPN)	1,3-Diphosphoglyceraldehyde + DPN $\rightleftharpoons$ 1,3-diphosphoglycerate + $\text{DPNH}_2$	(151, 211)
10	Nonenzymatic	1,3-Diphosphoglycerate + ADP $\rightleftharpoons$ 3-phosphoglycerate + ATP	(151, 211)
11	Triose mutase (protein-M*)	3-Phosphoglycerate $\rightleftharpoons$ 2-phosphoglycerate	(134)
12	Enolase (protein-Mg)	2-Phosphoglycerate + $\text{H}_2\text{O} \rightleftharpoons$ phosphoenolpyruvate	(126, 213)
13	Phosphopyruvate phosphatase (protein-ADP-Mg)	Phosphoenolpyruvate + ADP $\rightarrow$ pyruvate + ATP	(41, 115, 166)

M\* = metal.

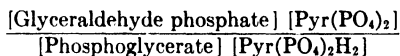
poor (Lipmann's terminology (119)) phosphate bond ester requiring the action of an enzyme, phosphorylase, the second leads to the formation of diphosphoglyceraldehyde and is nonenzymatic. In reaction 4 (fructose 6-phosphate + ATP  $\rightleftharpoons$  fructose 1,6-diphosphate + ADP) and reaction 5

(hexose + ATP  $\rightleftharpoons$  glucose 6-phosphate + ADP) phosphate is transferred from the energy-rich phosphate bond in adenosinetriphosphate through enzymatic reactions. In reaction 10 (1,3-diphosphoglycerate + ADP  $\rightleftharpoons$  3-phosphoglycerate + ATP and reaction 13 (phosphoenolpyruvate + ADP  $\rightarrow$  pyruvate + ATP) the energy-rich phosphate bond is restored to adenylic acid and thus adenosinetriphosphate is reformed; the first is a nonenzymatic reaction because of the lability of the phosphate group linked to the carboxyl residue and is the reason for the so-called "compulsory coupling between oxidation-reduction and phosphorylation"; the last is enzymatic and is the only reaction presumably irreversible. In reaction 2 (glucose 1-phosphate  $\rightleftharpoons$  glucose 6-phosphate) and in reaction 11 (3-phosphoglycerate  $\rightleftharpoons$  2-phosphoglycerate), both enzymatic, there is intramolecular migration of phosphate groups.

A number of the enzyme systems which catalyze these reactions have been isolated and purified, all of them being made up of specific proteins, most of them combined to magnesium and adenylic acid. Thus, phosphorylase is a protein-Mg-adenylic acid (70); phosphoglucomutase (48), enolase (213) and probably triose mutase (134) are protein-Mg compounds; phosphopyruvate phosphatase is a protein-ADP-Mg (166); phosphohexokinase and hexokinase seem to be protein-ATP compounds; zymohexase is, according to Green, *et al.* (83), a protein with no prosthetic group; phosphoglyceraldehyde oxidase, the oxidation ferment of Warburg and Christian (211), is a protein-Pyr(PO<sub>4</sub>)<sub>2</sub>; the prosthetic groups of isomerases (triose- and hexose-) are unknown. In summary, the series of eleven enzyme systems of the first phase of fermentation is made up mostly of specific metalloproteins with, in some instances, the addition of adenylic acid or its polyphosphates. In Warburg's enzyme there is addition of a sluggish reversible oxidation-reduction system.

**Equilibrium.**—In the enzymatic hydrolysis of glycogen or starch by amylase the reaction seems to be largely irreversible. The introduction of phosphate into the carbohydrate molecule brings forth an important property, that of reversibility, shared by all the reactions except the last, formation of pyruvate. The concentrations of the reacting systems, of ions which accelerate or retard the establishment of equilibrium, the hydrogen-ion concentration, and the temperature are factors which determine the value of the equilibrium constants, and hence the rate and the orientation of the different reactions. At constant temperature and pH value, the concentration of phosphate and of glucose 1-phosphate determines the equilibrium of the reaction: glycogen + H<sub>3</sub>PO<sub>4</sub>  $\rightleftharpoons$  glucose 1-phosphate. At 25° and pH 7, equilibrium is reached in reaction 1 with 77% glycogen

and 23% glucose 1-phosphate; in reaction 2, with 5% glucose 1-phosphate and 95% glucose 6-phosphate; in reaction 3, with 30% fructose 6-phosphate and 70% glucose 6-phosphate (46). Temperature plays an important role in some of these reactions and is negligible in others, thus altering in opposite directions their orientation. For example, while equilibrium in reaction 2 is almost independent of temperature changes, reaction 6 (fructose 1,6-phosphate  $\rightleftharpoons$  dioxyacetone phosphate + 3-glyceraldehyde phosphate) is greatly affected by it, the equilibrium value  $K$  varying from  $1.8 \times 10^{-4}$  at  $-7^\circ$  to  $2.2 \times 10^{-2}$  at  $70^\circ$  (136). The influence of phosphate and  $H^+$ -ion concentration on the equilibrium value of the oxidation-reduction system represented by the over-all reaction:



is also considerable. According to Warburg and Christian (212), at optimum phosphate concentration, equilibrium was established at  $pH$  7.4 when  $\text{Pyr}(\text{PO}_4)_2$  was half reduced ( $E_h$  about  $-0.292$  volt), while at  $pH$  8.45 it was established at 91.4% reduction of  $\text{Pyr}(\text{PO}_4)_2$  ( $E_h$  about  $-0.382$  volt). The factors which affect the value of the equilibrium constant in these enzymatic reactions are considerably increased in the living cells by the interposition of semi-permeable membranes, viscosity, and among vertebrates by the presence of the hormonal controlling mechanism.

**The Role of Adenosine and Guanidine Phosphates.**—In the series of reactions presented in Table I, ATP acts as P mediator in four of them. In vertebrate muscle and in nerve there is another substance of similar properties, creatine, and in invertebrates, arginine. In Table II is presented the series of reactions in which these P mediators transfer energy-rich phosphate bonds. Muscle myosin seems to contain three mechanisms of P transfer: Native myosin, which breaks  $\text{ATP} \rightarrow \text{ADP} + \text{HPO}_4$  (59, 147); myosin II, precipitated myosin, which takes another P:  $\text{ADP} \rightarrow \text{adenylic acid} + \text{H}_2\text{PO}_4$  (9); and, finally, myokinase, which seems to catalyze the reaction:  $2\text{ADP} \rightleftharpoons \text{ATP} + \text{adenylic acid}$  (42, 99). Those reactions which are produced in the muscle fibers are essential for the process of muscular contraction, where, as observed by Lohmann (125), the splitting of ATP precedes that of creatine phosphate. The adenylypyrophosphatase activity of muscle is associated with the myosin fraction of muscle proteins, for after purification of myosin by repeated reprecipitation the protein was still just as active in splitting off one  $\text{PO}_4$  group from ATP but had lost entirely the power shown by the mash or by once precipitated myosin of converting ADP into adenylic acid. Myosin I seems to be a

protein-Ca compound, for  $\text{Ca}^{++}$  is the most efficient activator; myosin II contains an adenosinediphosphatase activated by  $\text{Mn} > \text{Mg} > \text{Ca}^{++}$ . It can readily be seen that so rich a mechanism for the liberation of energy-rich phosphate bonds will contribute these labile phosphates to the prosecution of the reactions in Table I. Thus, in the presence of  $\text{CH}_2\text{ICOOH}$ , reactions 10 and 12 take place with creatine (166) instead of ADP. The equilibrium constants of the reactions

$$k_1 = \frac{\text{ATP} \times \text{Arg}}{\text{ADP} \times \text{Arg P}}; k_2 = \frac{\text{ATP} \times \text{Cr}}{\text{ADP} \times \text{Cr P}}; k_3 = \frac{\text{ADP} \times \text{Cr}}{\text{Ad} \times \text{Cr P}}$$

were measured by Lehmann (113).

TABLE II

REACTIONS OF ADENOSINE AND GUANIDINE PHOSPHATES ON ADDITION OF THOSE IN TABLE I

No.	Enzyme system	Reacting systems	References
1	Myosin I	$\text{ATP} \rightarrow \text{ADP} + \text{H}_2\text{PO}_4$	(59, 147)
2	Myosin II	$\text{ADP} \rightarrow \text{adenylic acid} + \text{H}_2\text{PO}_4$	(9)
3	Myokinase	$2 \text{ADP} \rightleftharpoons \text{ATP} + \text{adenylic acid}$	(99)
4	Adenyltriphosphatase	$\text{ATP} + \text{creatine} \rightleftharpoons \text{ADP} + \text{creatine phosphate}$	(115, 125)
5	Adenyltriphosphatase	$\text{ATP} + 2 \text{creatine} \rightleftharpoons \text{adenylic acid} + 2 \text{creatine phosphate}$	(114)
6	Adenyltriphosphatase	$\text{ATP} + \text{arginine} \rightleftharpoons \text{ADP} + \text{arginine phosphate}$	(113)

ATP and the phosphorus transfer enzymes seem to be universally distributed, for they exist in animal tissues, in bacteria, in yeast, in plants, and in fungi; their importance in the efficient transfer of energy is indeed great. (See Lipmann's excellent review (119).) In fact, by transferring P reversibly to creatine and arginine they contribute energy for muscular contraction; they contribute energy for the reversible phosphorylations of fermentation (Table I); and they contribute energy for the aerobic phosphorylations and synthesis, thus linking catabolic and anabolic processes of carbohydrate metabolism. If the existence of energy-rich phosphorylated intermediates in carbohydrate oxidation (acetyl phosphate, phosphosuccinate; phosphooxaloacetate; phospho- $\alpha$ -keto- $\beta$ -carboxyglutarate) is demonstrated, the role of ATP in transferring energy from these labile compounds would be established.

Creatine, which is abundantly distributed in muscle and nerve (69, 135), has been found by Torres (199a) in mammalian spermatozoa; whether the



breakdown of creatine phosphate provides the energy for their motility remains to be demonstrated. Arginine in invertebrate muscle seems to play the same role as creatine (10, 160). Arginine can be regarded as the more primitive substance, creatine being derived from it. In fact, Schoenheimer (176) demonstrated that the synthesis of creatine in the animal body may occur by transmethylation from arginine in the presence of glycine and methionine. The replacement of arginine by creatine in the evolution from invertebrate to vertebrate is a matter of great interest. The replacement starts among the invertebrate group of *Echinoides* (sea urchins) and *Enteropneusta* (*Balanoglossus*), which seem to be the connecting link; the larvae themselves contain only arginine, while creatine appears after the metamorphosis. In vertebrate muscle over half of the total creatine is combined with P (69). In invertebrates, practically no free arginine exists in the fresh oxygenated muscle (133, 135). The transformation of arginine into creatine was of significance in the evolution process because it increased the efficiency of this reservoir of energy ( $\Delta H$  of phosphocreatine  $\rightleftharpoons$  creatine +  $H_3PO_4$  = -10,700 cal;  $\Delta H$  of phosphoarginine  $\rightleftharpoons$  arginine +  $H_3PO_4$  = -7700 cal (142); there are not yet reliable data for the calculation of  $\Delta F$  values) and hence the efficiency of the cell machine when burning carbohydrate as fuel.

**Distribution of the First Phase of Fermentation.**—The series of reactions tabulated in Table I was studied in tissue and yeast extracts and in isolated enzymes prepared from such extracts. From many quarters doubt has been raised as to the application of those findings to the events *actually* occurring in living cells. Furthermore, the assumption that the process is universal has also been challenged. Needham and his co-workers (148–150), in their series of papers on the intermediary carbohydrate metabolism in embryonic life, came to the conclusion that in chick embryo there are two separate routes of carbohydrate breakdown: (1) nonphosphorylating glycolysis, very active and closely bound to the cell structure; and (2) phosphorylating glycolysis, similar to that of muscle. The reasons for this conclusion were that P transporting coenzymes were present in very small amounts, that the rate of glycolysis was not affected by the complete removal of phosphate, and that there was inhibition of glycolysis by *l*-glyceraldehyde, which they considered a specific inhibitor of nonphosphorylating glycolysis. It is known that phosphopyridine nucleotides are easily dephosphorylated in tissues and adenosine is easily deaminated; destruction of these coenzymes might have been the reason for the failure of Needham, *et al.*, to find the phosphorylation series in embryo glycolysis. In fact, Meyerhof and Perdigon (140) demonstrated the presence of diphos-

phopyridine nucleotide and adenosinetriphosphate in extracts of mouse and chick embryo which readily converted a mixture of pyruvate and hexose diphosphate into lactic acid and phosphoglyceric acid on addition of the necessary coenzymes. The slow decomposition of hexose diphosphate is no contradiction, because (see Table I) the rate of oxidation-reduction of triose phosphate depends on the simultaneous phosphorylation of the adenylic acid system which transfers P to the acceptors, glucose, hexose monophosphate and creatine. Regarding the inhibition of glycolysis by glyceraldehyde, it is due to inhibition of hexokinase activity (186) and is no argument against glycolysis with phosphorylation. The same assumption of two mechanisms of carbohydrate breakdown was made for brain glycolysis (5), an assumption based mainly on the fact that the rate of glycolysis was higher in the presence of glucose than with glycogen, and on the inhibiting effect of *l*-glyceraldehyde. The rapid destruction of adenosine and pyridine nucleotide observed by Mann and Quastel (129) was again the reason for this conclusion, for it has been shown that brain (62) has the same glycolytic mechanism as that of muscle (131). Fermentation by phosphorylation has been demonstrated in retina extracts (189-191, 102), in tumor extracts (35), in cartilage (76) and in the liver (164). In spite of the accumulating evidence in favor of phosphorylated fermentation in animal tissues, there are still voices raised against transferring conclusions drawn from experiments in tissue extracts to the living animal; and Sacks (174) has challenged its applicability even to muscle, a tissue from which most of the enzymes described in Table I were isolated. The decrease in creatine phosphate, with corresponding increase in inorganic phosphate, and the accumulation of hexose monophosphate found in muscle contraction under anaerobic conditions are considered by him objections against the Embden-Meyerhof scheme of Table I. However, synthesis of phosphocreatine is a process connected with respiration (26); the reaction  $\text{ATP} + \text{creatine} \rightleftharpoons \text{creatine phosphate} + \text{ADP}$  is reversible and under anaerobic conditions the reaction proceeding to the right will produce a diminution of available ATP required for the phosphorylation of hexose monophosphate. The validity of Sacks' experiments with radioactive phosphorus has already been challenged by Lipmann (119) and by Kalckar (98). From a methodical consideration of all the papers published on this subject, it may be concluded that in animal tissues the first phase of carbohydrate fermentation proceeds strictly *via* phosphorylation, a mechanism which allows a greater utilization of the energy derived by the breakdown of the molecule into the  $\text{C}_3$  compounds.

According to Negelein and Brömel (152) the activating protein of alcohol oxidase in yeast is able to oxidize dihydroxyacetone, dihydroxyacetone

phosphate and glyceraldehyde when acetaldehyde reduction is stopped by bisulfite fixation. Electron transfer occurs through diphosphopyridine nucleotide, and glycerol is the end product. The affinity of the activating protein is, however, 20,000 times greater for acetaldehyde than for dihydroxyacetone, the fastest reacting substance of the three intermediates above mentioned.

Very little is known about the mechanism of carbohydrate fermentation in invertebrates and protozoa. Anaerobic glycolysis and the presence of carbohydrates in sea urchin eggs was demonstrated in 1928 (168), and recently the presence of pyruvate (18). Diphosphopyridine nucleotide and its synthesis by *Chilomonas paramecium* was demonstrated by Hutchins, *et al.* (89). It may be expected that there will be found among the lower invertebrates, especially among the protozoa, fermentation mechanisms requiring no phosphorylation, for Reiner, Smythe and Pedlow (172) found that glucose is broken down anaerobically by *T. equiperdum* to pyruvate and glycerol,  $C_6H_{12}O_6 \rightarrow CH_3COCOOH + C_3H_8O_3$ , while *T. lewisi* gives first 1 molecule of succinate and 1 molecule of glycol (or acetaldehyde +  $H_2O$ ),  $C_6H_{12}O_6 \rightarrow HOOCCH_2CH_2COOH + C_2H_6O_2$ . This is followed under anaerobic conditions by the formation of acetic acid and ethyl alcohol. Since such decomposition is anaerobic, it may be concluded that the first phase of fermentation, glucose  $\rightarrow$  pyruvate, exists in *T. lewisi*. Pyruvate may then condense with  $CO_2$ , as postulated by Searle and Reiner (177), to give oxaloacetate which through reduction *via* malate will end in succinate. Part of the pyruvate molecule is decarboxylated and the acetaldehyde is dismutated to ethyl alcohol and acetic acid. Whether the first phase of fermentation proceeds *via* phosphorylation is not known. That protozoa utilize carbohydrates has been established by numerous investigators through growth experiments; it remains to investigate whether the carbohydrate breakdown proceeds through reversible phosphorylations.

The necessity of preliminary phosphorylation of carbohydrate in yeast has also been challenged, although many of the reactions of Table I were performed with yeast extracts. Nilsson and Alm (157), for example, maintain that fermentation reactions in yeast cells proceed at first without previous phosphorylations, and that the phosphorylation reactions occur later; in fact, they found that dried yeast cells could ferment glucose in the absence of phosphate with the formation of a nonfermentable  $C_3$  compound. On the other hand, Macfarlane (130) maintains the existence of phosphorylated fermentation in living yeast. Although the presence of non-phosphorylated fermentation in living yeast cells has not yet been established, it cannot be denied that the accumulation of phosphate esters

in yeast extracts is an artifact brought about through cell disintegration. Werkman and his co-workers (see Werkman's review (215a)) have contributed most to the demonstration that the reversible processes of phosphorylation do exist in bacteria. In *Esch. coli*, for example, all the individual reactions of Table I have been demonstrated to take place (56, 187, 201) and in *Streptococcus fecalis* there was observed on the fermentation of glucose a parallel decrease of inorganic P and increase of organic P, which appeared primarily as a Ba-soluble, alcohol-insoluble fraction (162). But even in *Esch. coli* there are present enzyme systems (glyoxalase) which might be taken as evidence for the existence of fermentation without phosphorylation (188). There is some indirect evidence in favor of the existence of nonphosphorylated fermentation in bacteria. The ability of *Propionibacterium pentosaceum*,\* grown in fluoride-containing media, to ferment glucose (217), and the lack of fluoride inhibition of glucose fermentation by certain bacteria\* (17) are indication that the anaerobic fermentation of glucose in bacteria might also take place without phosphorylation. Glucose fermentation without phosphorylation might exist among saprophytic bacteria, soil bacteria, for example, which have the property of easily forming new enzyme systems to utilize the organic substance in which they are grown.

We are indebted to James and his co-workers, and to Hanes for much progress on understanding the mechanism of carbohydrate breakdown in plants. The existence of the first phase, carbohydrate  $\rightarrow$  pyruvate, has been shown conclusively by James and his co-workers in a series of papers dealing with the metabolism of barley. Pyruvic acid was formed by barley sap from glucose in the presence of adenylic acid (91, 94), and the necessity of phosphorylation for carbohydrate breakdown was also demonstrated by studying the ratio of CO<sub>2</sub> formation to inorganic P disappearance (92, 93). The importance of phosphorylation for the carbohydrate metabolism of plants has been corroborated by the excellent work of Hanes (78) on the breakdown and synthesis of starch by a phosphorylase quite similar to Cori's phosphorylase but isolated from potato and pea seeds. Starch or any saccharide composed of  $\alpha$ -glucopyranose units linked in positions 1 and 4 are readily phosphorylated by plant phosphorylase (pea, potato) with the formation of a glucose 1-phosphate identical with the Cori ester obtained by phosphorylation of glycogen. The presence of hexose diphosphate, which is readily used by plants (3), is

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\* According to Warburg and Christian (213) fluoride inhibits enolase by forming a protein Mg-fluorophosphate complex compound.

another evidence for carbohydrate phosphorylation. However, much remains to be investigated; for example, the existence of Warburg and Christian's reaction (reactions 8, 9 and 10 of Table I) has not yet been demonstrated. Very little is known about the carbohydrate fermentation mechanism of green algae; they are able to ferment glucose (68) but the mechanism of this fermentation is unknown.

Whether the first phase of carbohydrate fermentation exists among the lower fungi has not yet been demonstrated. Glucose is fermented by fungi. Nord and his colleagues (158, 159), who have studied carefully the carbohydrate fermentation in *Fusaria*, demonstrated that there exist in the multicellular fungi two types of fermentation: without previous phosphorylation during the first days of growth, and fermentation through phosphorylation afterward, the exact mechanism of the nonphosphorylated fermentation being unknown.

If fermentation by phosphorylation in the first phase has been found in animal tissues, yeast, plants, bacteria, fungi, can it be concluded that the enzyme systems are identical throughout the living organisms? Since the first enzyme system, phosphorylase, is the only enzyme system which has been isolated in animal tissues, yeast and higher plants, a comparison of the properties of phosphorylase isolated from them may be useful. Phosphorylase from animal tissues is a protein-adenylic acid complex (70), the presence of small amounts of glycogen being necessary to start the reaction toward glycogen formation. Phosphorylase from potato seems to need no adenylic acid (73). However, while liver phosphorylase gives glycogen which is indistinguishable from natural glycogen in its chemical properties (49), muscle phosphorylase gives a polysaccharide very similar in structure

to plant starches (8). The equilibrium value  $k = \frac{c \text{ inorg. P}}{c \text{ Cori ester}}$  varies with the pH value; while it changes from 5.7 at pH 6 to 2.9 at pH 7.55 in muscle phosphorylase (49), it changes in potato phosphorylase from 10.8 at pH 5 to 3.1 at pH 7 (79). This discrepancy reflects some real difference in the reaction occurring in the plant and animal systems. Phosphorylase from yeast seems to be also different from the other two phosphorylases; it requires no adenylic acid, and the  $k$  value at pH 7 is 5.2 (103). Furthermore, the muscle of embryo and the newly born animal has less phosphorylase activity than that of the adult animal (143).

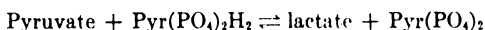
It may be concluded from this review that phosphorylative fermentation represents an advanced stage in the evolutionary process of carbohydrate metabolism and that nonphosphorylative fermentation seems still to exist among the fungi, yeasts and some bacteria.

## 2. Second Phase: Anaerobic Decomposition of Pyruvate

The discovery of alcohol as an end product of some fermentations is indeed very old (see Neuberg's review); the discovery of lactic acid came many years later (Bernard (30)). When an increased lactate concentration was found in the contracting muscle, attention was so much focused on this end product of fermentation that much effort has been wasted and still is being wasted in futile attempts to correlate lactate formation with carbohydrate oxidation, with carbohydrate synthesis. Lactate and alcohol are end products of the anaerobic phase of some carbohydrate fermentations. Whatever is gained in the reduction of pyruvate is lost in the reverse process, oxidation of lactate. In most aerobic cells, moreover, the anaerobic breakdown stops at the pyruvate stage, pyruvate being then metabolized through a variety of channels, of which lactate is only a *cul de sac*, a wasteful step observed in abnormal conditions, when there has been a temporary or permanent interruption of optimal aerobic conditions.

While the first phase of the fermentation process has been found in almost all living organisms, the second phase is extremely varied. It ends in lactic acid formation in animals and some bacteria; in alcohol formation in plants, yeast, fungi and some bacteria; and in a large number of other products. Perhaps the gradual replacement of  $\text{CO}_2$  by phosphate as the organisms proceed further in the evolutionary stages of biochemical efficiency, accompanied by the disappearance of carboxylase responsible for the production of  $\text{CO}_2$  from pyruvate, is the cause for this change.

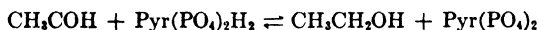
Pyruvate in the presence of the enzyme system lactate oxidase (in animals, protein-DPN) is reversibly reduced to lactate:



the  $\text{Pyr}(\text{PO}_4)_2\text{H}_2$  molecule being provided by reaction 9 of Table I. Since the  $E'_0$  of  $\text{Pyr}(\text{PO}_4)_2$  is, at pH 7,  $-0.28$  volt (34) and that of lactate  $-2e \rightleftharpoons \text{pyruvate}^- + 2\text{H}^+$  is  $-0.180$  volt (21) in the absence of oxygen, pyruvate will be completely reduced to lactate if no other oxidation-reduction systems of more positive potential are present. In the living organism, the ratio:  $\frac{\text{pyruvate}}{\text{lactate}}$  will depend on the pH value, on the extent of reaction 9 which provides  $\text{Pyr}(\text{PO}_4)_2$ , and on the extent of oxidation, amination and condensation reactions undergone by pyruvate. This ratio seems to remain in resting men more or less fixed according to Friedemann and Barborka (66).

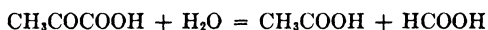
The end products of fermentation in protozoa are almost unknown, difficulty of growing them in pure culture being the obstacle.

In yeast, the main product of fermentation is alcohol. Pyruvate is irreversibly decarboxylated to acetaldehyde and  $\text{CO}_2$ , and acetaldehyde is reduced by alcohol oxidase—Warburg's reducing enzyme of fermentation (205) (protein-Pyr( $\text{PO}_4$ )<sub>2</sub>). This reduction is also reversible:



Here also since the  $E'_0$  value of the system acetaldehyde  $\rightleftharpoons$  alcohol is, at pH 7,  $-0.165$  volt (16) reduction of acetaldehyde will be complete in the absence of oxygen. There is in yeast slight formation of lactate (85).

The greatest diversity in the anaerobic fermentation of carbohydrate is seen in bacteria. Moreover, the quantities of these different products are not at all constant, but may vary markedly under the influence of the external conditions under which fermentation takes place. Pyruvic acid may end with the formation of formic and acetic acid (22, 155):



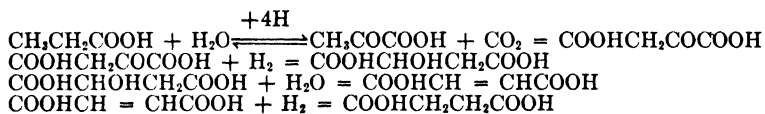
It may end by dismutation in acetic and lactic acids (23, 109):



It may give by condensation acetylmethyl-carbinol (74, 180):



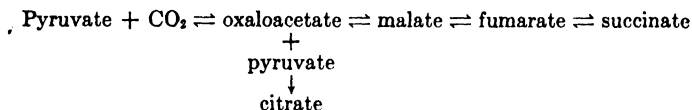
It may give by fixation of  $\text{CO}_2$  a series of end products, by reactions whose mechanism has been so fruitfully investigated by the laboratories of Werkman and co-workers (see Werkman and Wood's review (216)) and the California workers (202): for example, formation of succinic acid (218); of propionic acid (38):



Evidence for the reversibility in bacteria of Wood and Werkman's reaction ( $\text{CH}_3\text{COCOOH} + \text{CO}_2 \rightleftharpoons \text{COOHCH}_2\text{COOH}$ ) has been provided by Krampitz and Werkman (106) with the striking demonstration of the enzymatic  $\beta$ -decarboxylation of oxaloacetate heretofore purely hypothetical. The existence of this Wood and Werkman's fixation reaction in animal tissues has not yet been demonstrated. Block and Barron (unpublished experiments from this laboratory) attempted to orient the metabolism of pyruvate toward oxaloacetate formation by increasing the  $\text{CO}_2$  tension, and by diminishing the temperature to  $20^\circ$  (to avoid oxaloacetate decom-

position). Although there was vigorous pyruvate utilization in liver slices of rat and pigeon, no oxaloacetate was detected.

In plants, there is also abundance of carboxylase the presence of which has been demonstrated by numerous investigators (36, 88, 25); as a consequence, alcohol is one of the end products of carbohydrate fermentation. There is also every reason to assume that the fixation reaction of Wood and Werkman plays an important role in the metabolism of pyruvate. The presence in plants of malic, succinic, fumaric, citric acids may start with this fixation reaction:



The presence of carboxylase in fungi has been demonstrated (199), and the formation of alcohol seems a general phenomenon. Here, too, it may safely be predicted that the fixation reaction of Wood and Werkman provides the route for the formation of the citrate, malate, fumarate, succinate found in cultures of fungi (175, 181, 199).

There are some bacteria which give as main products of fermentation, both alcohol and lactic acid (65) and others which give mainly alcohol (199). A careful study of the fermentation mechanisms of these microorganisms might provide the discovery of the factors which changed one mode of fermentation into the other.

### III. Oxidative Breakdown of Carbohydrate

### 1. Without Previous Fermentation

There is now accumulated incontrovertible evidence against the view that carbohydrate utilization starts only after its anaerobic breakdown to pyruvate: in animal tissues, the oxidation of fructose by the brain (121), the continued respiration of tissues after inhibition of glycolysis by  $\text{CH}_3\text{ICOOH}$  (14) ( $\text{CH}_3\text{ICOOH}$  inhibits reaction 9 of Table I); in yeast, the oxidative breakdown of phosphogluconic acid (58), the direct oxidation of maltose (200), the uninterrupted respiration in the presence of concentrations of  $\text{CH}_3\text{ICOOH}$  enough to inhibit fermentation (128); in bacteria, the oxidation of glucose and hexose phosphates by microorganisms which do not ferment glucose (17), the oxidation of gluconic to  $\alpha$ -ketogluconic acid (120); in fungi, the direct oxidation of glucose to gluconic acid (31), to kojic acid (195), to keto-gluconic acid (84).

### The enzyme system for the direct oxidation of glucose—glucose oxidase



(protein-Pyr(PO<sub>4</sub>)<sub>3</sub>)—has been found in animals in the liver (80), and abundantly distributed in molds (144, 196). The enzyme system for the direct oxidation of hexose monophosphate (protein-Pyr(PO<sub>4</sub>)<sub>3</sub>-alloxazine mononucleotide-cytochrome c) exists in yeast, in some bacteria, and possibly in other organisms. Hexose monophosphate can be oxidized indeed by a series of dehydrogenations and decarboxylations (51, 117, 208, 209), giving some justification to Engelhardt's opinion that "hexose monophosphate represents the point where the fate of the hexose molecule is determined and the paths of respiration and fermentation diverge" (57). The enzyme system for the oxidation of  $\alpha$ -phosphoglycerol (71), which might oxidize carbohydrate before pyruvate formation, exists in animal tissues, plants and bacteria.

More data are needed for a comparative study of these nonfermentation oxidations. Vigorous in molds and in some saprophytic bacteria, they seem to lose importance as the cells advance in their evolutionary process, and their presence in higher organisms might represent a relic of ancestral processes which becomes operative when the more efficient phosphorylative mechanisms have been interrupted.

## 2. Carbohydrate Breakdown via Pyruvate

There is no intermediary substance produced during the metabolism of foodstuff that possesses the reactivity of pyruvate, and is able to take part in such a variety of reactions. Pyruvate is thus the hub toward which converge carbohydrate, fats and proteins in their catabolic and anabolic reactions. The manifold reactions tabulated in Table III have been found to occur in animal tissues, yeast, bacteria, fungi and plants; and the orientation of these reactions will of course be determined in the living cell by the different factors controlling the velocity of the different enzymatic reactions. The pressure of oxygen, the concentration of phosphate and other electrolytes, the concentration of the reacting substances, of the prosthetic groups of the activating proteins, the presence in higher organisms of the regulating mechanisms which control the speed of reactions—leaving aside the influence of foreign agents—all will determine the orientation and the rate of the metabolism of pyruvate, and hence of carbohydrate. The oxygen pressure, which in a contracting muscle, for example, changes continuously, has a great influence on determining the orientation, because pyruvate (like the other  $\alpha$ -keto acids) is utilized in the presence, as well as in the absence of oxygen. The ratio of  $\frac{\text{pyruvate used in O}_2}{\text{pyruvate used in N}_2}$ , i. e., the oxydis-

mutation coefficient, changes from tissue to tissue. In bacteria there is even greater variation (Table IV), for there are bacteria which utilize pyruvate only in the presence of oxygen, and bacteria which have oxydismutation coefficients lower or greater than unity. The conclusion is inescapable that any single scheme devised to explain pyruvate utilization in *living cells* has per force to be inapplicable.

TABLE III

PROSTHETIC GROUPS OF ACTIVATING PROTEINS OF ENZYME SYSTEMS FOR PYRUVATE METABOLISM

No.	Prosthetic group of activating protein	Reversibility	Nature of reaction	End product determined	References
1	Diphosphopyridine nucleotide	Reversible	Oxidation-reduction	Lactate	(207)
2	Flavin dinucleotide	Reversible	Oxidation-amination	Alanine	(210)
3	Unknown (not (1) or (4))	Reversible	Transamination	Alanine	(24)
4	Diphosphothiamine	Irreversible	Decarboxylation	Acetaldehyde + CO <sub>2</sub>	(127)
5	Diphosphothiamine	Irreversible	Oxidation	Acetate + CO <sub>2</sub>	(23, 118)
6	Diphosphothiamine	Irreversible	Dismutation	Lactate + acetate + CO <sub>2</sub>	(23, 118, 179)
7	Diphosphothiamine	Irreversible	Dismutation	Acetate + formate	(22)
8	Diphosphothiamine	Irreversible	Condensation	Acetylmethylcarbinol + CO <sub>2</sub>	(74, 180)
9	Diphosphothiamine	Irreversible	Condensation	Carbohydrate	(24)
10	Diphosphothiamine	Irreversible	Condensation	Citrate	(24)
11	Diphosphothiamine	Irreversible	Condensation	Acetoacetate	(24)
12	Diphosphothiamine	Irreversible	Condensation	Succinate	(24)
13	Diphosphothiamine	Irreversible	Condensation	$\alpha$ -Ketoglutarate	(24)
14	Unknown	Reversible?	CO <sub>2</sub> fixation	Oxaloacetate	(106)

The excellent work of Lipmann (118) on the mechanism of pyruvate oxidation by acetone-dried preparations of *Lactobacillus Delbrückii* opened up an avenue of ample perspectives toward the understanding of the role of phosphorylations in respiration. Oxidation of pyruvate through an intermediary phosphate to acetyl phosphate, and transfer of the phosphate bond to ADP for the formation of ATP ready to provide its high energy of dephosphorylation for the production of other chemical reactions was partially confirmed by Banga, Ochoa and Peters (13) in work with brain extracts. Whether the oxidation of pyruvate proceeds *via* acetyl phosphate in all living cells is not yet known. There are indications, indeed, that there

are cells which oxidize pyruvate vigorously in the absence of phosphate. In fact, the rate of oxidation of pyruvate by thoroughly washed *M. piltonensis* was found to be the same in the presence and in the absence of phosphate (unpublished experiments). Furthermore, the enzyme system for the oxidation of pyruvate is also different even among bacteria, for the oxidation of pyruvate in *L. Delbrückii* proceeds by reduction of alloxazine dinu-

TABLE IV

PYRUVATE UTILIZATION OF LIVING CELLS IN THE PRESENCE AND IN THE ABSENCE OF OXYGEN

Oxydismutation coefficient =  $\frac{\text{Pyr. utilized in O}_2}{\text{Pyr. utilized in N}_2}$  Pyruvate in cu. mm. per mg. per hr.  
(data from unpublished experiments from this laboratory and from Barron and Lyman (23)).

Cell	Pyruvate utilization		
	In O <sub>2</sub>	In N <sub>2</sub>	Oxy-dism. coeff.
Mammalian tissues (rat)			
Heart	9.7	5.2	1.86
Diaphragm	8.1	4.1	1.98
Abdominal muscle	3.0	1.7	1.76
Liver	6.5	6.0	1.08
Kidney	15.4	7.4	2.08
Brain	9.0	7.0	1.28
Spleen	5.1	2.6	1.96
Testis	9.0	8.2	1.10
Skin	3.0	2.8	1.07
Invertebrate tissue			
Sea urchin egg non-fertilized	0.02	..	..
Sea urchin egg fertilized	0.12	..	..
Bacteria			
<i>Streptococcus hemolyticus</i>	10.5	12.7	0.82
<i>Staphylococcus albus</i> No. A 43	23.8	12.6	1.89
<i>Staphylococcus albus</i> No. A 4, 8 years old	29.0	0	∞

cleotide and oxygen while its oxidation by gonococci requires the cytochrome system (15). Pyruvate oxidation in sea urchin eggs seems to play an essential role in providing the energy necessary for fertilization, for the amount of pyruvate utilized by the eggs during the first hours of fertilization is five times greater than utilized by the nonfertilized eggs (18). The oxidation of pyruvate is not a general reaction; many bacteria, yeasts, plants, molds, protozoa do not oxidize it. It seems that tissues having a

powerful carboxylase system do not oxidize pyruvate; as carboxylase disappears from cells diphosphothiamine combines with the activating protein of pyruvate oxidase and the pyruvate oxidation system starts to function.

**The Role of Sluggish Oxidation-Reduction Systems in the Oxidation of Pyruvate.**—In 1928, Quastel and Wooldridge (170) suggested for the first time that biological oxidations could be carried by the mediation of coupled reactions with other systems. In 1935 Borsook (33) gave some examples of the realization in biological systems of this thermodynamic possibility by oxidizing sluggish systems of negative oxidation-reduction potential with sluggish systems of more positive potential through the mediation of electroactive systems of suitable potential, the extent of oxidation of the sluggish system A by the sluggish system B being determined by the equation

$$E'_{0A} - E'_{0B} = \frac{RT}{nF} \ln \frac{(A \text{ ox}) (B \text{ red})}{(A \text{ red}) (B \text{ ox})}$$

where the intermediary catalyst and dissociation constants have been ignored for the sake of simplicity. In living cells flavoproteins act as the electron mediators between the reversible systems. The oxidation of phosphoglyceraldehyde by pyruvate (52) with the formation of glycerophosphate and lactate is an example. A further and more important application was that postulated by Szent-Györgyi (194), where pyruvate oxidation was assumed to be performed by electron mediation through the systems oxaloacetate  $\rightleftharpoons$  malate, fumarate  $\rightleftharpoons$  succinate, cytochromes  $\rightleftharpoons$  O<sub>2</sub>. Later Krebs added citric acid to these systems. Szent-Györgyi's postulate has solid thermodynamic bases, for both these systems have potentials suitable for carbohydrate oxidation ( $E'_0$  of oxaloacetate  $\rightleftharpoons$  malate at pH 7,  $-0.102$  volt;  $E'_0$  of succinate  $\rightleftharpoons$  fumarate, 0 volt), also the electron mediators Pyr(PO<sub>4</sub>)<sub>2</sub>, flavoproteins and the cytochrome system. The oxidation-reduction potential of the system isocitrate  $\rightleftharpoons$   $\alpha$ -keto- $\beta$ -carboxyglutarate is unknown. Krebs' "citric acid cycle" is ingenious and attractive, for it provides a path for the gradual withdrawal of electrons and of CO<sub>2</sub> molecules for the complete breakdown of pyruvate, and every effort made for its verification is indeed commendable. Starc, *et al.* (183), from kinetic experiments doubt the validity of the scheme. Wood and Werkman, *et al.* (219) (after Evans and Slotin (63) demonstrated the presence of radioactive carbon in  $\alpha$ -ketoglutarate produced in liver suspensions from pyruvate), studied carefully and completely with the aid of heavy carbon the state of all the postulated intermediary substances in the formation of  $\alpha$ -ketoglutarate, and proposed a new scheme shown in Fig. 1 as slightly altered by Krebs (108). In this scheme, citrate becomes the blind alley that lactate is, and

the extent of its formation would be determined by the activity of aconitase. The introduction of the reversible system isocitrate  $\rightleftharpoons$  *cis*-aconitate needs to be further studied. Isocitrate is oxidized by the protein-TPN system (2); and the introduction of this system would require either the transfer of electrons from  $\text{Pyr}(\text{PO}_4)_3\text{H}_2$  to another unknown system<sup>1</sup> or through the alloxazine mononucleotide of Haas, Horecker and Hogness (77) to cytochrome system and  $\text{O}_2$ . The electron transfer, shown in Krebs' scheme (107), from isocitrate to oxaloacetate is contrary to established fact because the reduction to malate is performed by  $\text{Pyr}(\text{PO}_4)_3\text{H}_2$ . It becomes then essential to verify whether the oxidation of isocitric acid is catalyzed by the system protein- $\text{Pyr}(\text{PO}_4)_3$  + protein-alloxazine mononucleotide + cytochrome c + cytochrome oxidase. If the introduction of the reversible system isocitrate  $\rightleftharpoons$   $\alpha$ -keto- $\beta$ -carboxyglutarate as another catalyst for car-

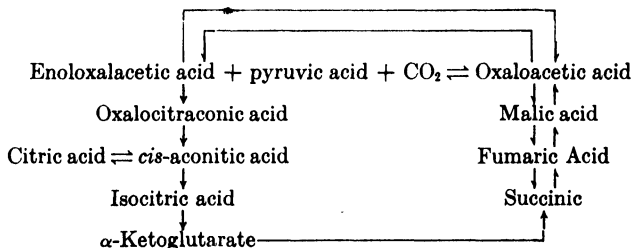


Fig. 1.—The citric acid cycle without citric acid. Wood and Werkman's modification of Krebs' modification of Szent-Györgyi's cycle.

bohydrate oxidation is demonstrated, it must be a relatively new feature, mostly confined to muscle (like creatine) because even among mammals this enzyme system is absent in some tissues. Among invertebrates very little has been done; the eggs of sea urchins did not utilize citrate. Most bacteria utilize citrate at a much lower rate than either glucose, pyruvate or succinate, and a large number of them do not utilize it (Table V). Higher plants (61, 144) and yeast (32), but not molds (196) contain the enzyme but in none of them is carbohydrate utilization increased on addition of citrate.

Regarding the electron-mediator role of the other systems, oxaloacetate  $\rightleftharpoons$  malate, and fumarate  $\rightleftharpoons$  succinate, there is evidence that they might act as such much more widely than the isocitrate  $\rightleftharpoons$   $\alpha$ -keto- $\beta$ -glutarate system. The distribution of the first system has not been studied methodically; the distribution of the second is almost universal among living cells possessing cytochrome c. Sea urchin eggs, for example, which have cytochrome oxidase and no cytochrome c (105), are unable to oxidize suc-

inate (12); yet they oxidize pyruvate. The catalytic role of these systems has been strikingly shown by Ochoa (161) and by Cori and his co-workers (43), in their work on phosphorylated oxidations.

Regarding the pathway of pyruvate oxidation in plants, and the role of the enzymatic sluggish reversible systems, much remains to be done. The mechanism of formation of citric, malic, fumaric, succinic and oxalic acids, which accumulate in many plants (29, 169), must also be studied in the light of new accumulated knowledge; the formation of these organic acids might easily be explained by postulating the existence in plants of the en-

TABLE V

COMPARATIVE RATES OF UTILIZATION OF PYRUVATE, CITRATE AND  $\alpha$ -KETOGLOUTARATE BY TISSUES AND CELL SUSPENSIONS

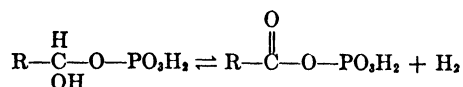
Figures give cu. mm. per mg. per hr. (unpublished).

Tissue or cell suspension	Pyruvate	Citrate	$\alpha$ -Ketoglutarate
Mammalian tissue			
Kidney	17.3	5.6	6.8
Heart	13.2	4.3	2.7
Liver	6.1	1.5	1.8
Testis	10.8	0.4	1.1
Diaphragm	7.3	1.6	1.3
Rectus	4.1	0.6	1.0
Skin	3.0	0	0
Invertebrate cells			
Sea urchin fertilized	0.1	0	0
Bacteria			
<i>Ps. aeruginosa</i>	50.0	0.5	1.5
<i>G. tetragina</i>	10.4	0.2	12.0
Gonococci	22.1	0	0

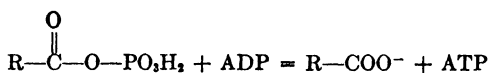
zyme system responsible for Wood and Werkman's  $\text{CO}_2$ -fixation reaction, but proof is lacking. Moreover, most plants contain no  $\alpha$ -ketoglutarate (169), and succinic oxidase has been found absent in some germinating seeds (Bartlett's unpublished observations in this laboratory). Pyruvate can be made to accumulate in saps from leaves of barley plants by addition of acetaldehyde (94), a fact which is taken by James and co-workers as indication that pyruvate metabolism in this plant is oriented mainly through decarboxylation. This conclusion is hardly valid because of the likelihood of loss of many oxidation enzymes during the process of sap formation (freezing and thawing method).



The step of biological importance in this reaction is the formation of an energy-rich phosphate bond during the oxidation-reduction process,



and the transfer of this phosphate bond to other compounds able to form again energy-rich phosphate bonds, the energy of which may then be used for the activities of the cell:



By assuming the entry of phosphate into compounds with carbonyl groups (a reaction more general and not confined to Warburg's reaction), Lipmann discovered the formation of acetyl phosphate. The oxidation of  $\alpha$ -ketoglutarate to succinate by  $\alpha$ -ketoglutarate oxidase requires an enzyme containing diphosphothiamine (20) like that of pyruvate; it is therefore logical to assume that the intermediate product of this oxidation might be the energy-rich phosphate bond succinyl phosphate; the oxidation of isocitrate to  $\alpha$ -keto- $\beta$ -carboxyglutarate might also bind phosphate at the carbonyl group; and finally enoloxaloacetate might form phosphoenoloxaloacetate. The entry of phosphate into these oxidation-reduction systems, with formation of a series of labile phosphate groups ready to phosphorylate adenylic acid and creatine, would explain the necessity of these systems in phosphorylated oxidations. None of these phosphorylated compounds, however, has been isolated from reactions in living cells.

The necessity of phosphorylations in the oxidation of carbohydrates by organisms other than vertebrates has not yet been shown, although, as usual, the universality of its existence is assumed. Its importance in muscle activity has been proved, for here the phosphocreatine decomposed during the anaerobic process is reformed through the aerobic phosphorylations (26) and the hypothetical labile phosphate compounds already mentioned will transfer P to creatine, thus reforming phosphocreatine. James and Arney (92) believe that, since the presence of phosphate increases the respiration of young barley seedlings, there is a link between respiration and phosphorylation. It should be noted that fermentation by phosphorylation and aerobic metabolism of pyruvate without phosphorylation will give also the results obtained by James and Arney. It is well to remember that plants display a great versatility in promoting interconversion of carbohydrates, many of which are known exclusively as products of plant metab-



olism. Furthermore, the activity of zymases in many plants is so vigorous (198) that hydrolytic splitting might not be an uncommon mechanism in plants.

It must have been noticed that phosphate concentration exerts a powerful action on the rate of many of the reactions in the anaerobic and the aerobic phase. Thus, to go back to Table I, the orientation of reaction 1 depends both on the concentration of inorganic P and that of hexose ester; as a consequence, when the concentration of inorganic phosphate in the cells is increased, glycogen might be expected to be broken down. If glucose 1-phosphate formed is removed by other enzymes, the breakdown of glycogen will continue until the concentration of inorganic phosphate has returned to the base level. When the concentration of phosphate is decreased, reaction 1 will orient toward glycogen synthesis. The position of the equilibrium constant of reaction 9 depends also on the concentration of inorganic phosphate because of the phosphorylation of 3-glyceraldehyde phosphate and on the presence of P acceptors such as adenylic acid. The rate of oxidation of pyruvate depends also on the concentration of phosphate. Other ions play a prominent role (Mg,  $H^+$ , Mn, Ca).

#### 4. *Isolated Enzyme Systems, Extracts, Ground Tissues, Tissue Slices, the Living Cell*

In the living cell which is a heterogeneous system, the varieties of proteins taking part in enzymatic reactions are separated from each other by semi-permeable membranes, and the whole is separated from the environment by another semi-permeable membrane. If living cells join together to form the society of multicellular organisms, the complexity of the chemical activities increases again by the interaction of the intercellular milieu, and the introduction of regulating mechanism; a hopeless task it would be indeed to study the mechanism of these biochemical activities without studying the individual chemical activities isolated from each other. But once the different enzyme systems are isolated, their equilibrium constants and kinetics determined, their distribution throughout the living kingdom established, their interactions studied, and the interaction of the intracellular and extracellular fluids determined, there still remains the task of verifying whether in the living cells these chemical activities *do* take place as found in these solutions. In isolated enzyme systems we are dealing with solutions; in extracts and ground tissues we are dealing with broken cells with the architecture ruined to varied degrees, with the membranes destroyed, and proteins unfolded or precipitated. As a consequence, some reactions, only possible at the sur-

face of the membranes, might disappear altogether or diminish their rate. Others, which do not normally take place because of interposition of these membranes or other steric hindrances, will appear, especially those coupled oxidation-reductions (see Korr's excellent review on oxidation-reduction in heterogeneous systems (104)) which might take part in carbohydrate breakdown. It is well to remember, therefore, that the "enzyme chemist" has not solved nor will he solve the chemical activities of the living cell if he does not constantly have in mind what the living cell is, and what controlling mechanisms are added to those cells as they go higher in their organization, in their evolutionary processes. A few examples might be given in support of these warnings. On work with tissues it is alleged that blood serum is the physiological fluid to use when determining *in vitro* their biochemical activities; such an assumption is, of course, false because the composition of the blood serum is not identical with that of the intercellular spaces of the particular tissue. Furthermore, *in vitro* work in closed vessels with animal tissue slices means a change from an open, circulating fluid where the ionic environment is kept constant to a closed system with fixed environment. According to Hastings and his co-workers (81), the synthesis of glycogen of rat liver is increased fourfold if the liver slices are suspended in a solution containing K and Mg at the concentrations found in the intracellular fluid, in the presence of glucose; glycogen formation in liver slices suspended in solutions resembling the extracellular fluid was very small. As Hastings very properly expresses:

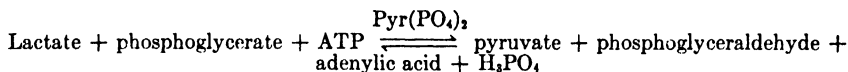
"The maintenance of a particular intracellular ionic environment is probably as important for the normal activity of certain intracellular enzymes as is the maintenance of a particular extracellular ionic environment for the maintenance of the normal activity of the cell as a whole" (81, p. 121).

It is very well known that the respiration of tissue slices proceeds smoothly when suspended in Ringer solutions; when the tissue is ground, Ca must be eliminated and the concentration of NaCl must be diminished in order to have suspensions with constant  $O_2$  uptake (55). The metabolism of pyruvate in liver slices is partly oriented toward condensation, dismutation and transaminations; in ground liver, the metabolism is mainly oriented toward oxidation (unpublished experiments); furthermore, while the production of acetoacetate from pyruvate in rat liver slices disappears when the tissue is ground, it increases on grinding in the liver of pigeon (24). The respiratory quotient of kidney slices in Ringer-phosphate is 0.86 (7); kidney extracts oxidize glucose to  $CO_2$  and  $H_2O$  with a R. Q. of 1. The oxidation of *D*-amino acids by *D*-amino acid oxidase (protein-alloxazine dinucleotide) is insensi-

tive to HCN; in kidney slices this oxidation is inhibited by HCN. Glucose oxidase (protein-Pyr(PO<sub>4</sub>)<sub>3</sub>-flavoprotein) has been isolated from the liver (80); up to now the direct oxidation of glucose by liver slices has not been demonstrated. When Dakin discovered *glyoxalase* in 1913 (50) and Neuberg (155) at the same time formulated his scheme of carbohydrate fermentation by glucose splitting to methylglyoxal, fermentation *via* methylglyoxal was found to take place in more and more cells. Nobody now speaks of the role of glyoxalase in the breakdown of carbohydrate by the liver.

#### IV. The Anabolic Process: Synthesis of Carbohydrate

As has already been stated, all the series of reactions going from glycogen to pyruvate are reversible except No. 13 (Table I), the dephosphorylation of phosphopyruvic acid. Energy is therefore required for reversing this reaction. The synthesis of glycogen starting from any of the other reactions might be accomplished by only adjusting the different factors determining the equilibrium reactions so that the reactions might go from right to left and upward. The demonstration of the continuous reversibility of these reactions from glucose to glycogen has been given by Colowick and Sutherland (44), who converted glucose to glycogen *in vitro* in the presence of the different enzyme systems, phosphorylase, phosphoglucomutase, and isomerase obtained from muscle, plus hexokinase from yeast and adenosinetriphosphate. The reversal of the reactions from 8 to lactic acid was first demonstrated by Green, *et al.* (72); in this reaction, as shown by Meyerhof, *et al.* (139), phosphopyruvic acid originating by equilibrium reaction from phosphoglycerate transferred the energy-rich P bond to phosphoglyceraldehyde, the over-all reaction being:



The phosphoglyceraldehyde formed presumably condenses to hexose diphosphate. The formation of phosphopyruvic acid from C<sub>4</sub> dicarboxylic acids has also been shown, the reaction being an oxidative one (97). Resynthesis of carbohydrate from pyruvic acid and those C<sub>4</sub> carboxylic acids was first demonstrated by Elliott, *et al.* (54); and the direct participation of pyruvate in this synthesis was shown by Barron and Lyman (23) in experiments with kidney slices from thiamin-deficient rats. Carbohydrate synthesis in these experiments was considerably below normal; it increased to normal values on addition of thiamin, thus proving that synthesis under

those conditions was brought about by pyruvate condensation. The mechanism of this condensation process, for bacteria, was postulated by Wood and Werkman as a  $\text{CO}_2$  fixation reaction ( $\text{pyruvate} + \text{CO}_2 = \text{oxaloacetate}$ ). The existence of this reaction in animal tissues was made probable by the discovery of Evans and Slotin (63), who showed that when pyruvate is incubated with ground pigeon liver and  $\text{C}^{14}\text{O}_2$ , the radioactive carbon is found in the carboxyl group of  $\alpha$ -ketoglutarate; under the same conditions, Wood, *et al.* (219), found also malate and fumarate containing heavy carbon. Finally Lipmann (119) postulated the formation of phosphoenol-oxaloacetate, which would readily give phosphopyruvate. The striking observations of Hastings and his co-workers on the formation of radioactive glycogen by rats when fed lactate or glucose and with  $\text{NaHO}_3\text{C}^{11}$  injected intraperitoneally have given strong support to the possibilities and postulates mentioned above. In the first series of experiments (182) in which lactate was fed and  $\text{NaHO}_3\text{C}^{11}$  injected, they found that the liver glycogen contained 0.65% of the total radioactive carbon injected; on feeding glucose and injected  $\text{NaHO}_3\text{C}^{11}$  they found that the figure rose to 0.87%, while the radioactivity of muscle glycogen was 0.035% (204). When lactate containing radioactive carbon in  $\alpha$ - or  $\beta$ -position was fed the glycogen contained 3.2% of the radioactivity while lactate with radioactive carbon in the carboxyl group contained only 1.6% (203). Their conclusions are in full agreement with the different facts observed and summarized above, and give the strongest indirect evidence of the existence in mammals of Wood and Werkman's fixation reaction.\* They are well aware that to postulate glycogen formation *only via*  $\text{CO}_2$  fixation to pyruvate would be a fallacy in view of the reversibility of so many of the reactions of carbohydrate breakdown. Extending to their experiments Schoenheimer's conclusions on the dynamic equilibrium of reversible reactions (176) they agree that "the existence of equilibria between the various chemical components involved in the carbohydrate system causes a distribution of labeled carbon atoms introduced at any one state, into all the molecular species involved." It must be recalled that since glycogen synthesis does not occur in the absence of oxygen, the energy required for this synthesis is provided by the energy-rich phosphate bonds formed during the oxidation of carbohydrate. An

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\* There has recently appeared from the laboratory of Hastings (Buchanan, J. M., Hastings, A. B., and Nesbitt, F. B., *J. Biol. Chem.*, **145**, 715 (1942)), a preliminary note showing conclusively that glycogen synthesis in the liver occurs *via* pyruvate condensation. With rabbit liver slices, in a medium containing radioactive carbon as  $\text{KHC}^{14}\text{O}_3$ , 1.67 per cent of the total radioactive carbon was found in the synthesized glycogen, approximately 12 per cent of it being derived from (+4) carbon.

appraisal of the contribution of these labile substances has to wait until the existence of the labile phosphorylated  $C_4$  and  $C_5$  compound has been disclosed.

The synthesis of carbohydrates from fats probably occurs through the link between acetoacetic acid and pyruvic acid. Weil-Malherbe (215), in fact, observed the synthesis of carbohydrate by the kidney incubated with acetoacetate.

Very little is known about the mechanism of carbohydrate synthesis in other organisms. In yeast, it possibly proceeds as in mammalian tissues. In plants, where  $CO_2$  plays an important role, the dark reduction of  $CO_2$  builds the series of small carbon-containing molecules; the photochemical reactions play in these  $CO_2$  reactions (reduction, fixation) the same role as that of the energy-rich phosphate bonds, that of increasing the efficiency of the  $CO_2$  reduction. It must be recalled that starch is formed by phosphorylation. Very little is known about the mechanism of synthesis of the variety of polysaccharides produced by molds, or about the mechanism of carbohydrate synthesis from acetate so readily performed by protozoa.

**The Role of Glutathione in Carbohydrate Metabolism.**—Glutathione, the  $-SH$  group containing tripeptide, which after its discovery by Hopkins (86) was thought to be one of the catalysts for cellular oxidations, has had an accidented career. That it does not belong to the group of electroactive oxidizing catalysts which constitute the last reversible step in the series of reactions between substrate and molecular oxygen was shown by its inertia toward molecular oxygen. And the function of this powerful reducing substance universally distributed among living cells, existing in greater concentration in embryonic cells, where the synthetic processes occur with greater intensity, is still shrouded in mystery. The demonstration of the presence of active sulfhydryl groups in the activating protein of succinoxidase by Hopkins and Morgan (87), the demonstration by Rapkine (171) that the activating protein of phosphoglyceraldehyde oxidase (Warburg's oxidation enzyme of fermentation) contains also active  $-SH$  groups, and by Lehmann (112) that phosphoglucomutase is inactivated by GSSG and reactivated by GSH, made imperative a systematic investigation of the other enzyme systems taking part in the breakdown and synthesis of carbohydrate. If the activating proteins of these systems contained also  $-SH$  groups essential for their activity, the role of glutathione in carbohydrate metabolism would become one of primary importance, for it would be that of reactivating  $-SH$  groups whenever there was oxidation or transformation of these groups. The presence of such  $-SH$  active groups became more probable on recalling Barron's observations (15) on the la-

bility of pyruvate oxidase ( $\alpha$ -ketonoxidase); the enzyme was promptly destroyed by 2,6-dichlorophenol indophenol, lost its activity on standing, although in the absence of oxygen the activity lasted longer. The presence of active —SH groups was detected by the use of mercaptan-forming compounds, alkyl-forming compounds and mild oxidizing agents. On inactivation of the enzyme by arsenicals or chloromeric benzoic acid (mercaptan-forming compounds), there was in every case reactivation by glutathione. The following enzyme systems (the activating protein) were found to contain —SH groups essential for enzyme activity: pyruvate

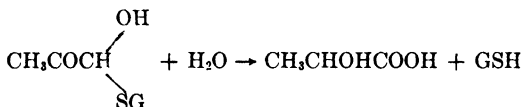
TABLE VI

ACTIVE —SH GROUPS IN THE ACTIVATING PROTEINS OF ENZYME SYSTEMS TAKING PART IN CARBOHYDRATE METABOLISM

Enzyme system	Active —SH groups	References
Phosphorylase	—	(112)
Phosphoglucomutase	+	(112)
Phosphoglyceraldehyde oxidase	+	(171)
Pyruvate oxidase	+	This laboratory (unpublished)
Pyruvate condensation	+	This laboratory (unpublished)
Lactic oxidase	—	This laboratory (unpublished)
Carboxylase	+	(75)
Alcohol oxidase (liver)	—	This laboratory (unpublished)
Alcohol oxidase (yeast)	+	This laboratory (unpublished)
Malic oxidase	+	This laboratory (unpublished)
Citric oxidase	—	This laboratory (unpublished)
$\alpha$ -Ketoglutarate oxidase	+	This laboratory (unpublished)
Succinoxidase	+	(87)
<i>d</i> -Amino acid oxidase	+	This laboratory (unpublished)

oxidation, pyruvate condensation (synthesis of carbohydrate, synthesis of acetoacetate synthesis of  $\alpha$ -ketoglutarate), malic oxidase,  $\alpha$ -ketoglutarate oxidase, and *d*-amino acid oxidase. Of all these enzymes it is the activating protein for pyruvate activation (oxidation, condensation) which seems to contain most of the active —SH groups at the outer surface of the protein, being, as a consequence, extremely susceptible to the action of mild oxidizing agents. In Table VI are tabulated all the enzyme systems concerned with carbohydrate metabolism where the existence of —SH groups has been properly investigated. It can be seen from this table that the presence of —SH groups is essential for the activity of the activating proteins for fermentation (phosphoglucomutase, phosphoglyceraldehyde oxidase), for oxida-

tion (pyruvate oxidase, succinoxidase, malic oxidase,  $\alpha$ -ketoglutarate oxidase), for condensation, acting possibly on Wood and Werkman's reaction —CO<sub>2</sub> fixation (carbohydrate synthesis), for amination (*d*-amino acid oxidase). The facile inactivation of all these enzyme systems by reagents combining with or oxidizing —SH groups and the complete reactivation by the addition of glutathione are offered as evidence for the important function of glutathione in carbohydrate metabolism. Glycolysis, direct pyruvate oxidation, pyruvate fixation of CO<sub>2</sub>, pyruvate oxidation through Szent-Györgyi's cycle and carbohydrate synthesis are all processes whose optimal activities are maintained by glutathione whenever there has been impairment of the active —SH groups. There has been as yet no comparative study of this important problem. Suffice it to say that Chaix and Fromageot (39) have offered evidence for the presence of —SH groups in one or more of the enzyme systems for fermentation in *Propionibacterium pentosaceum*; that the presence of —SH groups in the enzyme from one cell does not mean that this enzyme will contain active —SH groups in all cells (yeast alcohol oxidase contains active —SH groups while liver alcohol oxidase does not contain them); and finally, that if glyoxalase takes part in carbohydrate metabolism, the role of glutathione would be extended to this system because the formation of lactic acid from methylglyoxal is accelerated on combination with glutathione (95, 122):



The enzyme performs an inner Cannizzaro reaction with liberation of glutathione.

## V. Regulatory Mechanisms

The rate of reaction of isolated enzyme systems, as determined by numerous investigators, is extremely high when compared with the rate of respiration in living cells or multicellular organisms (Table VII). It must therefore be controlled in the living cell. The usual physicochemical factors which govern the rate of those reactions in solution, the disposition of these enzymes in heterogeneous systems, and the interposition of interfaces are the simplest mechanisms of regulation. Possibly many unicellular organisms possess only those mechanisms and well deserve to be called "pockets of enzymes." As the cell increases in efficiency, however, enzyme activities become controlled by specific controlling mechanisms

which in multicellular organisms freely circulate through the intercellular fluid and become distributed among the different cells of the organism. The nature of these specific regulatory mechanisms in bacteria and in plants is unknown. In invertebrates they become more differentiated. The respiration of sea urchin sperm, for example, is maintained at a low level under the regulating influence of some substance possessing  $-SH$  groups; on dilution or on addition of  $CH_2ICOOH$  or malonate (usual respiratory inhibitors), the regulatory system becomes inactivated and the respiration is increased by 100% (19). In vertebrates, these specific regulatory mechanisms increase in number and in specificity and control the rate of enzyme

TABLE VII

RATE OF REACTION OF SOME ENZYME SYSTEMS TAKING PART IN CARBOHYDRATE METABOLISM

( $K$  = number of molecules undergoing reaction per minute) compared to rate of respiration ( $K$  = moles  $O_2$  taken  $\times$  gm. dry weight  $\times$  min.)

Enzyme system	$K$	References
Catalase	$2.4 \times 10^6$ ( $0^\circ$ )	(184)
Peroxidase	$1.5 \times 10^6$ ( $20^\circ$ )	(100)
Cytochrome oxidase	$2.3 \times 10^6$ ( $20^\circ$ )	(206)
Zymohexase	$8.8 \times 10^3$ ( $38^\circ$ )	(83)
Phosphoglyceraldehyde oxidase	$2.0 \times 10^4$ ( $20^\circ$ )	(212)
Enolase	$1.0 \times 10^4$ ( $20^\circ$ )	(213)
Hexose monophosphate oxidase	$2.9 \times 10^4$ ( $38^\circ$ )	(153)
Acetaldehyde reductase	$2.9 \times 10^4$ ( $20^\circ$ )	(154)
Respiration		
<i>Azotobacter</i>	$7.15 \times 10^{-4}$	(214)
Retina (rat)	$2.28 \times 10^{-5}$	(178)
Liver (rat)	$1.04 \times 10^{-5}$	(173)

reactions by interaction among each other. In other words, these regulatory mechanisms, hormones, do not act by increasing the rate of reaction of an enzyme system but by releasing or increasing or maintaining the inhibiting action of the specific hormones. Insulin was prepared in pure, crystalline condition by Abel in 1927 (1); catalase was isolated in crystal form by Sumner in 1926 (192). In the meantime, the enzyme systems for the breakdown of carbohydrate are being isolated and purified, but in no chain of the series of reactions from carbohydrate to molecular oxygen does insulin appear (see Table I). The regulation of the blood sugar level must then be exerted by regulation of the rate of reaction of those enzyme systems con-



cerned with the formation and breakdown of glycogen to glucose, insulin being no component of an enzyme system. Gemmill, in fact (67), demonstrated an increase in glycogen synthesis *in vitro* by the diaphragm on addition of insulin; the increased deposition of glycogen was not associated with an increased oxygen consumption. However, Hechter, Levine and Soskin (82) point out that insulin enables the tissue to form glycogen at low concentrations of glucose; addition of insulin to a medium containing 400 mg. % of glucose had practically no effect on the glycogen formation. Whether insulin influences only the rate at which glucose penetrates the cell remains to be proved. The rate of synthesis of glycogen from glucose in the presence of phosphorylase, phosphoglucomutase, hexokinase, myokinase and ATP is independent of the presence of insulin (44). Adrenalin increases the rate of phosphorolysis of glycogen in muscle, there being an increased formation of glucose 1-phosphate and a decrease of inorganic phosphate; in the isolated enzyme system it has no effect at all (45). The corticosterones produced by the adrenal cortex form also part of the regulatory mechanism of carbohydrate metabolism. And the fundamental work which Houssay started in 1929 with his discovery that the removal of the pituitary from the diabetic dog produced a restoration of an apparently normal carbohydrate metabolism, gave the most striking evidence of the interaction of these regulatory mechanisms of carbohydrate metabolism in vertebrates. Thus, substances produced by the hypophysis, the pancreas, the adrenals, by interaction, by increasing or releasing specific regulations proper to one of them tend to maintain a regulated flow of energy and by thus doing increase the efficiency of the chemical activities of higher organisms.

## VI. Orientation of Reactions (the Pasteur Effect)

The cells belonging to the lowest group in the phylogenetic scale are undoubtedly those able to live and multiply in the absence of oxygen in which the energy requirements are met by fermentation processes. Fermentation is much the simpler of the two processes, but at the same time the less economical because the largest part of the energy of the carbohydrate molecule remains in the lactic acid or the alcohol molecule. The greater energy requirements of morphologically and physiologically complex organisms introduced atmospheric oxygen into the metabolism of living cells and thus respiration started. As soon as respiration appeared there arose the problems of orientation of reactions because most reactions are reversible and are interdependent. Aerobic cells in the absence of oxygen split

carbohydrate to lactic acid or alcohol; in the presence of air, oxygen is consumed,  $\text{CO}_2$  is produced, fermentation is stopped, and carbohydrate splitting diminished. The intimate relation of these two processes the genius of Pasteur discovered.

W. M. Clark expressed more than once his regret at having ever introduced the symbol  $rH$  into the field of oxidation-reductions because of continuous misuse and misinterpretation. If Pasteur could contemplate the misuses and misinterpretations of his discovery of the relation between respiration and fermentation, his regret would be as keen as Clark's. Burk in his presentation of "the Pasteur and neo-Pasteur effects" (37) has discussed them thoroughly and they need not be repeated here. It is, however, important to quote Pasteur:

"Free oxygen imparts to yeast an increased vital activity... If we supply yeast with a sufficient quantity of free oxygen for the necessities of life, nutrition and respiratory combustion, it ceases to be a ferment, that is, the ratio between the weight of the plant developed and that of the sugar decomposed is similar in amount to that in the case of fungi. On the other hand, if we deprive the yeast of air entirely it will multiply just as if air were present, although with less activity, and under these circumstances its fermentative character will be most marked; under these circumstances, moreover, we shall find the greatest disproportion, all other conditions being the same, between the weight of yeast formed and the weight of sugar decomposed... if free oxygen occurs in varying quantities, the ferment power of yeast may pass through all the degrees comprehended between the two extreme limits of which we have just spoken" (167, p. 259). "It must be borne in mind that the equation of a fermentation varies essentially with the conditions under which that fermentation is accomplished, and that a statement of this equation is a problem no less complicated than that of a living being" (p. 276).

Pasteur's foresight is strikingly confirmed as the mechanism of carbohydrate fermentation and oxidation becomes clarified. Throughout this article emphasis has been made on the integration of the series of reversible processes that take place during fermentation and oxidation, and the interrelation between both processes. All these interrelations explain the Pasteur effect: The link between fermentation and respiration (see Fig. 2) may be produced at the different steps of the series of reactions of carbohydrate metabolism which in anaerobic conditions end either in lactic acid or in alcohol. One of these steps may be the oxidation of hexose monophosphate, as Engelhardt thinks; another may be the oxidation of trioses through coupled oxidations with the sluggish enzymatic reversible systems (oxaloacetate  $\rightleftharpoons$  malate, fumarate  $\rightleftharpoons$  succinate. The link may occur in the competition for  $\text{Pyr}(\text{PO}_4)_3$  between lactate and oxaloacetate, as Ball pictured it (11). It may occur also after the formation of pyruvic acid, for part of it goes back to glycogen through condensation (aerobic resynthesis).



The orientation of reactions will be affected by the oxygen tension (101, 116); the temperature (110, 146, 53); the concentration of electrolytes (6, 40), of which that of phosphorus discussed by Johnson (96) is most important since its concentration determines the orientation of reaction 1, and 8 of Table I and that of phosphorylative oxidations. In vertebrates where the regulatory mechanisms become more complex with the appearance of the specific regulators, hormones, the orientation of reactions is also affected by their interaction (41). Figure 2 shows not only the complexity of the Pasteur effect, but also the futility of all those brave attempts that are still being made to pin down the Pasteur effect to a single cause. Of these attempts, that of Laser (111) (increased lactate formation without decrease in respiration observed in the presence of  $O_2:CO$  mixtures) has been studied by Stern and Melnick (185), who found that there was under such conditions formation of an Fe-porphyrin- $CO$  compound of absorption spectrum somewhat different from that of cytochrome oxidase. Without a careful study of the chemical reactions taking place in the retina when it is suspended in a solution saturated with  $O_2:CO$  (glucose, hexose phosphates, lactate, pyruvate) and a similar study of extracts from retina, no other conclusion is warranted than that inactivation with  $CO$  of an iron porphyrin compound produces in retina an increased acid formation, the  $O_2$  uptake remaining normal.

The Pasteur effect, inhibition of fermentation by  $O_2$ , visualized in Fig. 2, is a phenomenon present in all living cells able to ferment and oxidize carbohydrate; however, the complexity of the regulating mechanisms that link fermentation to respiration diminishes as the cells go down the phylogenetic scale.

## VII. Summary

Of all the foodstuffs, it is carbohydrate which is used the most for the energetics of cell activities. Unicellular organisms may break it by simple splitting, as in the Gay-Lussac equation, and may build carbohydrate by dark thermal reactions of  $CO_2$  reduction, of  $CO_2$  fixation. The appearance of oxygen in the air, and that of electroactive oxidation-reduction systems—alloxazins—made possible carbohydrate oxidation; either directly without previous phosphorylation as in fungi, or soon after phosphorylation as in some bacteria. Increased efficiency of fermentation was achieved by the introduction of energy-rich phosphate bonds which made possible the production of a series of reversible phosphorylations, and an intermediate reversible oxidation-reduction. In plants, increased efficiency in carbohydrate formation was made possible by the appearance of chlorophyll, which

increased the efficiency of  $\text{CO}_2$  reduction thus accelerating the series of dark, thermal  $\text{CO}_2$  reactions. Increased efficiency of carbohydrate oxidation started when the porphyrin nucleus of chlorophyll combined with iron, and the iron-porphyrin on combining with proteins gave a number of cytochrome components. A series of reversible oxidation-reduction systems, some of them sluggish, some enzymatic-sluggish, some electroactive, were thus interspersed between the carbohydrate and molecular oxygen, releasing energy by the smooth and gradual flow of electrons. Pyruvate, the hub toward which comes fermentation, from whence starts oxidation, was reduced under anaerobic conditions to lactic acid in animals, was decarboxylated to acetaldehyde and this reduced to alcohol in plants. In animals increased efficiency in carbohydrate formation was reached, by the replacement of chlorophyll by energy-rich phosphate bonds which make possible crossing the barrier interposed by the irreversible dephosphorylation of phosphopyruvic acid. Continuous interaction among these reversible oxidation-reductions, with electrons flowing to oxygen, with electrons flowing back to the synthesis of carbohydrate, was regulated by the establishment of regulatory mechanisms, which, on becoming more and more numerous as the activities of the multicellular organisms increased, reached the high degree of specificity and interaction in the hormones of animal organisms. As living cells march forward in their evolutionary process, their energy-producing mechanisms become more and more reversible, approaching the ultimate state, the state of equilibrium.

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# THE INTERMEDIARY STAGES IN THE BIOLOGICAL OXIDATION OF CARBOHYDRATE

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### Scope and Layout of the Article

The study of intermediary metabolism usually proceeds at two levels. At the first level the aim is to identify the intermediary compounds derived from the substrate molecule. When the intermediates have been discovered the second level can be approached—the study of the enzymes concerned with the activation of the intermediates. The present review is confined to the first level.

The article is in two parts. The first deals mainly with experimental observations and its object is to describe the basic facts on which the

present analysis of the biological oxidation of carbohydrate rests. It begins with a discussion of some principles of experimentation. This is followed by an account of the substances which have been found to be oxidizable in material metabolizing predominantly carbohydrate and which are therefore likely to be intermediates in the oxidation of carbohydrate. After this the biochemical reactions of these "possible" intermediates are described.

The second part deals mainly with theoretical aspects. It is based on the facts recorded in the first part and its object is to discuss the reaction schemes designed to describe the intermediate stages of carbohydrate oxidation.

The review centers around work on muscle tissue, the most thoroughly studied material so far, but reference to other materials, including micro-organisms, is frequently made and the similarities and differences between the various materials are discussed.

## I. Experimental Findings

### 1. *Methods of Experimentation*

**Chief Principle.**—Much importance is attached in the present analysis to "the fact that the body, though the seat of a myriad reactions and capable perhaps of learning, to a limited extent and under stress of circumstances, is in general able to deal only with what is customary to it" (Hopkins (1)). Relying on this fact one would expect that a tissue which under physiological conditions metabolizes only carbohydrate, will be able to deal only with carbohydrate and its metabolic derivatives when the range of its metabolism is examined by experiment. Conversely, if substances are added to such a tissue and are found to be metabolized they must be associated with carbohydrate metabolism. In practice, of course, there is no tissue and no cell whose metabolism is limited only to carbohydrate, but materials are available where the metabolism of carbohydrate decisively predominates. Chemical reactions found in experiments on such a material are *probably*, but not *certainly*, associated with carbohydrate metabolism.

The application of this principle has been a most profitable method of experiment in the study of carbohydrate oxidation. Alone and in conjunction with other methods (discussed in the following paragraphs) it has supplied the majority of the facts on which the present theory of the intermediary mechanism of carbohydrate oxidation is based.

**Muscle as Experimental Material.**—Striated muscle is a tissue in which carbohydrate is the predominant substrate for oxidations (see Gemmill (2)) and to which the above principle can therefore be applied. It has not been possible to demonstrate a direct oxidation of fat, fatty acids or glycerol in muscle (though it is probable that muscle can utilize fat indirectly, *e. g.*, after conversion into carbohydrate or into acetoacetate, but under suitable conditions the utilization of the latter is negligible compared with the oxidation of carbohydrate). Only three amino acids—glutamic acid, aspartic acid and alanine—are known to be oxidized in muscle and these amino acids are closely interlinked with carbohydrate metabolism, for their immediate breakdown products— $\alpha$ -ketoglutaric, oxaloacetic and pyruvic acids—are also intermediates in carbohydrate breakdown. It follows from these considerations that a substance which is *rapidly* metabolized in muscle may be expected to be associated with carbohydrate metabolism.

Muscle offers the further advantage of an exceptionally high rate of metabolism; for the higher this rate, the greater is in general the chance of obtaining chemical changes sufficiently large to be detected and measured. Different muscles show very considerable variations in their metabolic rates. The highest rates are found in mammalian heart and in pigeon breast muscle—the chief muscle employed in flying and therefore one of the most powerful muscles in the animal kingdom. These tissues have been extensively used in the study of carbohydrate oxidation.

**Tissue Preparations.**—Most of the recent work on the subject was carried out on isolated tissues which were either sliced, or minced, or extracted. Extracts of muscle and other tissues (Meyerhof (3)) contain the enzyme systems responsible for the anaerobic conversion of carbohydrate into lactic acid, but they are unable to bring about the major oxidations by molecular oxygen; oxidations frequently seem to be associated with “water-insoluble” constituents of the tissue and this is the reason why suspensions of minced tissue rather than extracts have commonly been used for the study of oxidative processes. Earlier investigators (Thunberg (4); Batelli and Stern (5)) chose solutions of phosphates as media. More recently (6, 7, 8) these have been replaced by isotonic and balanced salt solutions usually containing NaCl, KCl,  $\text{MgSO}_4$  or  $\text{MgCl}_2$  and phosphate buffer, *e. g.*, 1000 ml. 0.9% NaCl; 40 ml. 1.15% KCl; 10 ml. 3.84%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  and 300 ml. phosphate buffer, pH 7.4, the latter being prepared by dissolving 17.8 g.  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  and 20 ml. *N* HCl in 1 liter (8). Such a medium often gives higher metabolic rates than simpler media consisting of phosphate buffer only, or of isotonic sodium chloride solution (8, 9). Calcium salts inhibit oxidation in minced tissues and are therefore omitted.

While *breakdown* reactions such as the oxidation of carbohydrate appear to take place in minced tissues at rates occurring in the intact organ, minc-

ing, unlike slicing, more or less destroys the ability of the tissue to perform more complex reactions, *e. g.*, the synthesis of urea, or the Pasteur effect. It seems that mincing destroys, or damages, the mechanisms for the transmission of energy and thereby the ability to carry out syntheses.

An important factor in the use of minced tissue is the proportion of medium to tissue (10). If this proportion rises above 15 to 1 with pigeon breast muscle, and with some tissues above 3 to 1, the metabolic rates of the tissues fall. The decrease is approximately proportional to the degree of "dilution" of the tissue. This effect of dilution can be explained by the assumption that a reaction between more than two partners (*e. g.*, a ternary collision) determines the velocity of the metabolic reaction. The probability of a ternary collision decreases in proportion to the dilution. If one volume of enzyme solution is diluted with one volume of water the number of ternary collisions is halved in the total volume of the mixture. The effect of dilution is frequently that expected for a ternary collision.

Under optimal conditions the rate of respiration of minced tissues appears to be of the order of the maximal rate—as opposed to the rate of the resting tissue—occurring in the intact organ. Heart muscle provides an opportunity, not given by skeleton muscle, of comparing the relative rates in minced tissue and in physiologically active tissue. Smyth (11) found that the oxygen consumption of minced heart approaches the highest values for the heart working at maximal effort.

**Use of Inhibitors.**—"Specific" Inhibitors.—Enzyme inhibitors, especially "specific" inhibitors, are valuable reagents in the analysis of intermediary metabolism. An "ideal" specific inhibitor paralyzes one enzyme only, when added to a complex system of enzymes. As far as the enzymes taking part in carbohydrate breakdown are concerned, only one inhibitor is known which in certain circumstances comes within, or at least closely approaches, the definition of an "ideal" inhibitor. This substance is malonate (12). In low concentrations (0.001—0.005 *M*) malonate probably acts exclusively upon succinic dehydrogenase. At higher concentrations it may possibly interfere with other dehydrogenases (13, 14, 15) but this point requires further investigation.

If an enzyme, or enzyme system, which activates the breakdown of an intermediary metabolite is specifically inhibited the substrate of the enzyme must accumulate. Thus malonate causes an accumulation of succinate. The accumulation of the substrate is the most important criterion of a specific inhibition (of the type of enzyme in question). Specific inhibitions have often been claimed when it was found that a poison inhibits only one among a number of tested enzymes. Leloir and

Dixon (16), for instance, found that pyrophosphate inhibits succinic dehydrogenase, but has no effect on seven other dehydrogenases. They concluded that pyrophosphate probably resembled malonate and that its inhibitory effect on tissue respiration (Dixon and Elliott (17)) is due to the inhibition of succinic dehydrogenase. In fact, pyrophosphate, unlike malonate, does not cause an accumulation of succinate (18), and its inhibitory effects must therefore essentially be due to interference with other enzymes.\*

*"Selective" Inhibitors.*—The writer prefers to call inhibitors of the pyrophosphate type "selective" rather than specific inhibitors. The number of selective inhibitors is considerable. Iodoacetate, arsenite, fluoride, glyceraldehyde, phlorizine and tartronate belong to this group (for details the reader is referred to Cohen's review (20)).

If an ideal specific inhibitor inhibits a complex metabolic process it can be concluded that the inhibited enzyme takes part in the process. Thus the malonate inhibition of muscle respiration, or of pyruvate oxidation in certain tissues, indicates the participation of succinic dehydrogenase in these processes. In the case of a selective inhibitor no more than a suggestion can be derived from the inhibition of a complex process. For instance, if fluoride is found to be an inhibitor this merely suggests, but cannot be taken as conclusive evidence, that phosphorylated intermediates are involved. There is thus a difference in the nature of the conclusions which may be drawn from specific and from selective inhibitions and this justifies a distinction between the two types of inhibitors.

*Factors Affecting the Accumulation of Intermediates.*—The amounts of an intermediate which accumulate may be limited by the fact that the metabolism of some intermediates is interlinked with the metabolism of other intermediates. Thus a breakdown product of succinate—oxaloacetate—is required in those reactions which lead to the formation of succinate (as discussed later). Hence the accumulation of succinate will prevent the new formation of succinate. A similar case is that of phosphoglyceric acid formed during alcoholic fermentation. Fluoride causes an accumulation of this substance, but thereby also prevents its re-formation from glucose. In such cases the accumulation of larger quantities of the substrate will occur only if the loss of the normal precursor is compensated by the addition of a suitable substitute precursor, *i. e.*, fumarate in the first case, hexose-diphosphate in the second.

\* With reference to the statement of Adler, v. Euler, Günther and Plass (19) that pyrophosphate inhibits specifically isocitric dehydrogenase it may be mentioned that no isocitrate is found in pigeon breast muscle poisoned with pyrophosphate (18).



**Competitive Inhibitions by Substrates.**—If several oxidizable substances are present together in respiring material, they compete for the available oxygen (10, 21). This is illustrated by the following figures (21) which represent the oxygen uptake of bakers' yeast, suspended in 0.05 *M*  $\text{KH}_2\text{PO}_4$  at 17°.

Substrate added	$\text{QO}_2$
None	— 6.4
0.05 <i>M</i> glucose	—81.5
0.02 <i>M</i> <i>d,l</i> -lactate	—72.6
0.05 <i>M</i> glucose + 0.02 <i>M</i> <i>d,l</i> -lactate	—81.5

Whereas each substrate, when added alone, causes a large increase in the respiration, there is no summation of the individual effects when the substrates are present together. In other words, certain oxidizable substrates inhibit the oxidation of other substrates. If the second substrate is an oxidation product of the first, this inhibition will cause the accumulation of an intermediate. For example, high concentrations of citrate (0.05 *M*) inhibit the further oxidation of the primary oxidation product,  $\alpha$ -ketoglutaric acid. The latter accumulates and can be isolated as the dinitrophenylhydrazone. This principle is capable of general application. An excess of succinate causes an accumulation of fumarate and malate; an excess of oxaloacetate causes an accumulation of citrate,  $\alpha$ -ketoglutarate and succinate (18).\*

**Metabolic Quotients.**—It has proved convenient to many workers to express metabolic rates in terms equivalent to the expression  $\text{QO}_2 \left( \frac{\mu\text{l. O}_2 \text{ used}}{\text{mg. tissue} \times \text{hour}} \right)$  introduced by Warburg (22). All metabolites are expressed as  $\mu\text{l. gas}$ , 1 millimole being taken as equivalent to 22,400  $\mu\text{l.}$  The disappearance of a substance is expressed by the minus sign, the formation by the plus sign. This notation is used throughout this review.

## 2. Survey and Classification of Substances Metabolized in Muscle Tissue

Thunberg (4) was the first to examine systematically the oxidizability of organic substances in isolated animal tissues with the object of analyzing intermediary metabolism. Using the methylene blue technique and a micro-respirometer, he tested (1906–1920) the oxidation of over 60 organic substances, chiefly in muscle tissue. He discov-

\* A competitive inhibition explains the observation that malonate increases the oxidation of added citrate in muscle (31). In the absence of malonate citrate competes with its oxidation products, among them succinate and malate. Malonate removes the ability of these substrates to compete and thus facilitates the oxidation of citrate.

TABLE I  
LIST OF SUBSTRATES WHICH CAN BE METABOLIZED IN MUSCLE TISSUE  
(Excluding substances acting as catalysts or carriers, *e. g.*, coenzymes, adenylic acid, phosphagen. The figures in parentheses refer to references.)

I Glycolysis series (23) (rapidly metabolized)	II Citric acid series* (rapidly metabolized)	III Miscellaneous substances which are slowly metabolized†
Glycogen    Glucose-1-phosphate    Glucose-6-phosphate $\rightleftharpoons$ glucose    Fructose-6-phosphate    Fructose 1,6-diphosphate    $\alpha$ -Glycero-phosphate $\rightleftharpoons$ 3-glyceraldehyde-phosphate $\rightleftharpoons$ dihydroxyacetonephosphate    1,3-Diphosphoglyceralddehyde    1,3-Diphosphoglyceric acid    3-Phosphoglyceric acid    2-Phosphoglyceric acid    Phosphopyruvic acid    Pyruvic acid    Lactic acid	Citric acid    <i>cis</i> -Aconitic acid    Isocitric acid    $\alpha$ -Ketoglutaric acid $\rightleftharpoons$ 1(+) glutamic acid  Succinic acid    Fumaric acid    1(-) Malic acid    Oxaloacetic acid $\rightleftharpoons$ 1(-) aspartic acid  Pyruvic acid $\rightleftharpoons$ 1(+) alanine	Acetic acid (4) Butyric acid (4) Acetoacetic acid (24) $\alpha$ -Hydroxybutyric acid (4, 25) $\alpha$ -Hydroxyglutaric acid (4, 26) $\beta$ -Hydroxybutyric acid (4, 25) $\beta$ -Hydroxypropionic acid (25)

\* References are given later in this article.

† A number of further substances which according to Thunberg (4) cause a reduction of methylene blue in the presence of muscle are not included. They are formic acid, caproic acid, maleic acid, tartaric acid, tartaric acid, racemic acid and mesotartaric acid. A reduction of the dye suggests, but does not prove the oxidation of the substrate. To prove the oxidation it is necessary to demonstrate the disappearance of the substrate and the formation of an oxidation product. This has not been done in the case of the above acids.

ered the rapid oxidation of the salts of a number of acids, *viz.*, lactate, succinate, fumarate, malate, citrate and glutamate. Thunberg's results were confirmed and extended by Batelli and Stern (5) and later investigators. Batelli and Stern (5) appreciated the significance of these findings when they wrote, "Man kann annehmen, dass der die Oxydation dieser Säuren bewirkende Prozess mit dem der Hauptatmung der Gewebe identisch ist."

In subsequent years the work of Embden, Meyerhof, Robison, Cori and Cori and Warburg (see 23) on the anaerobic breakdown of carbohydrates added many further substances, mainly phosphorylated compounds, to the list of intermediary metabolites in muscle tissue.

TABLE II

EFFECT OF VARIOUS SUBSTANCES ON THE OXYGEN UPTAKE OF MINCED PIGEON BREAST MUSCLE

(The data refer to 4 ml. suspension; containing 270 mg. (fresh weight) muscle; medium: 30 parts 0.1 *M* phosphate buffer, pH 7.1, prepared by dissolving 17.8 g.  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  and 33.3 ml. *N* HCl in 1 liter, 5 parts 0.1 *M*  $\text{MgCl}_2$ ; 40° C.; final substrate concentration 0.01 *M*.)

Substrate added:	—	Citrate	<i>cis</i> -Aconitate	<i>dl</i> -Isocitrate	$\alpha$ -Ketoglutarate	Succinate
$\mu\text{l. O}_2$ absorbed:						
20 min.	445	720	667	637	632	745
40 min.	715	1173	1125	1035	1190	1262
60 min.	845	1593	1540	1435	1740	1740
120 min.	1005	2160	2040	1959	2620	2620
Substrate added:	Fumarate	1(−) Malate	Oxaloacetate	Pyruvate	1(+) Glutamate	1(−) Aspartate
$\mu\text{l. O}_2$ absorbed:						
20 min.	588	580	675	537	612	505
40 min.	1062	1080	1180	977	1090	945
60 min.	1612	1570	1670	1337	1570	1365
120 min.	2488	2455	2554	1982	2488	2098

These metabolites can be classified into three groups (Table I). The first comprises the substances arising from carbohydrate anaerobically. The second contains substances which can all be considered as arising during, or being connected with, the biological breakdown of citric acid. The substances listed in both these groups are relatively rapidly metabolized. The third group comprises compounds which are but slowly metabolized and are therefore not likely to be intermediates in the major metabolic processes of muscle tissue.

The addition of the substances of the first group to minced muscle suspensions frequently fails to increase the rate of the oxygen uptake, presumably because of the saturation of the tissue with carbohydrate or lactate. The substances of the second group generally increase the respiration of minced muscle suspension. An experiment illustrating the magnitude of the effect is shown in Table II. The effect of the added

substances often grows with time as the rate of respiration falls off in the control. The substances prevent to some extent this falling off and thus "stabilize" the initial rate.

Different investigators agree on the reality and on the magnitude of the effect shown in Table II, except in the case of citrate. Thunberg (27), Batelli and Stern (5) and Krebs and Johnson (28) found that citrate is rapidly oxidized; Breusch (29), Thomas (30), and Stare, Lipton and Goldinger (31) were unable to confirm this. These discrepancies are probably due to the fact that the effect of citrate, *cis*-aconitate and isocitrate and of other substrates depends, among other factors, on the ionic composition of the suspension medium (18). If plain phosphate buffer, or "phosphate saline," is used (15 parts to 1 part tissue) the effects of citrate are irregular; even strong inhibitions are found. These inhibitions and irregularities are abolished when magnesium chloride is added to the medium (see Table III). Magnesium ions are an essential constituent of the coenzyme complex of muscle respiration (Greville (7)). Added citrate "de-ionizes" magnesium (32), as it "de-ionizes" calcium, and thus causes inhibitions.

TABLE III

EFFECT OF THE MEDIUM ON THE RATE OF OXYGEN UPTAKE IN THE PRESENCE OF VARIOUS SUBSTRATES

(Minced pigeon breast muscle suspended in 14 parts medium; medium 1 contained 0.085 *M* phosphate buffer, pH 7.1; 0.03 *M* NaCl; 0.014 *M* MgCl<sub>2</sub>; medium 2 was "phosphate saline"; 40° C.; the data refer to 4 ml. suspension.)

Medium: Substances added (final concentrations 0.01 <i>M</i> ):	1 —	1 Citrate	1 1(+) Gluta- mate	2 —	2 Citrate	1(+) Gluta- mate	2 MgCl <sub>2</sub> : citrate
μl. O <sub>2</sub> absorbed:							
after 20 min.	607	850	705	605	565	810	760
after 60 min.	1302	2035	1965	1275	1140	2190	1660
after 120 min.	1585	2840	3160	1570	1230	3400	2390

Secondary inhibitions of various types are not infrequently encountered when substrates are tested for their oxidizability. In some instances high concentrations of the added substrates cause a fall in the oxygen uptake, presumably because of a competitive inhibition of dehydrogenases. This is true for oxaloacetate (33) and citrate (18) which inhibit succinic dehydrogenase and possibly other enzymes. Negative results or relatively low rates of reaction therefore do not always prove that an added substance cannot readily react. Only if a variety of different conditions has been tested is it justifiable to assume that the substrate in question cannot be metabolized or that its rate of metabolism is too low to allow the assumption that it is an intermediate in a major metabolic process.

### 3. Biochemical Reactions of Substances Metabolized in Muscle Tissue

This section deals with biochemical reactions of those substances listed in Table I which, on account of the rapid oxidation in muscle, may be expected to be intermediates in carbohydrate oxidation. The substances

of the "glycolysis series" are omitted from the following discussion, as their reactions have been fully reviewed elsewhere (23).

**Reactions of Citrate.**—*Conversion into  $\alpha$ -Ketoglutarate.*—Until 1937 the pathway of the citric acid breakdown was obscure. Earlier investigators expected that the biological oxidation might begin at the hydroxy group (like the oxidation by permanganate in acid solution) yielding acetone dicarboxylic acid and carbon dioxide. Experiments by Walker and his collaborators (34) suggest that this might, in fact, be the case in *Aspergillus niger* and *Pseudomonas pyocyanea* (though this has been questioned by Deffner (35)). It certainly does not apply to animal tissues or cucumber seeds (36, 37). Barthel (38) believed hydroxycitric acid ( $\text{HO}_2\text{C} \cdot \text{CH}_2 \cdot \text{C}(\text{OH})(\text{CO}_2\text{H}) \cdot \text{CH}(\text{OH}) \cdot \text{CO}_2\text{H}$ ) to be an intermediate in the bacterial decomposition of citric acid but this suggestion, it appears, has never been put to the test of experiment.

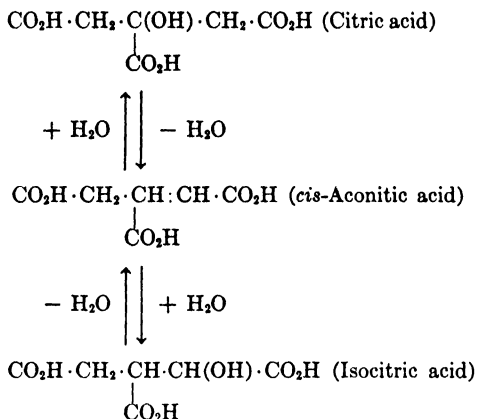
Wagner-Jauregg and Rauen (39) made considerable progress in the elucidation of the citric acid oxidation when they found in 1935 that 0.5 molecule  $\text{O}_2$  is used and 1 molecule  $\text{CO}_2$  is formed per molecule of citric acid oxidized by "citric dehydrogenase" from cucumber seeds, and, furthermore, that isocitric acid reacts at least as rapidly as citric acid while ketipic, acetoacetic, citraconic, oxaloacetic and acetic acids are inactive. The authors, however, failed to identify products of the oxidation. Martius and Knoop (40, 41) succeeded in solving this problem. Working with "citric dehydrogenase" from liver they discovered that the oxidation of citrate yields  $\alpha$ -ketoglutarate which was isolated as the 2,4-dinitrophenyl-hydrazone. This result was confirmed for muscle tissue (42).

The conversion of citrate into  $\alpha$ -ketoglutarate can best be demonstrated in "citric dehydrogenase" preparations (which appear to be unable to oxidize  $\alpha$ -ketoglutarate) or in tissue suspensions poisoned with  $\text{As}_2\text{O}_3$  (42). This inhibitor suppresses, though not quantitatively, the oxidation of  $\alpha$ -ketoglutarate, while it does not interfere with the reactions leading from citrate to  $\alpha$ -ketoglutarate. Some  $\alpha$ -ketoglutarate accumulates in the absence of poisons, when citric acid is added to respiring tissues in high concentrations (0.05–0.1 M) (18). These concentrations decrease the oxygen uptake and the oxidation of substrates other than citrate.

The formation of  $\alpha$ -ketoglutarate from citrate has so far been observed in animal (liver, muscle, kidney) and plant (cucumber seeds) material, but not in bacteria or in molds. Deffner (35), Franke and Deffner (43), Brewer and Werkman (44) and Slade and Werkman (45) have studied the anaerobic decomposition of citrate in *B. lactis* (*Aerobacter*) *aerogenes*, *Aerobacter indologenes* and *Streptococcus paracitrovorus* and have come to the conclusion that the cleavage of citrate into oxaloacetate and acetate ( $\text{CO}_2\text{H} \cdot$

$\text{CH}_2 \cdot \text{C}(\text{OH})(\text{CO}_2\text{H}) \cdot \text{CH}_2 \cdot \text{CO}_2\text{H} = \text{CO}_2\text{H} \cdot \text{CH}_2 \cdot \text{CO} \cdot \text{CO}_2\text{H} + \text{CH}_3 \cdot \text{CO}_2\text{H}$   
 is probably the first stage in the breakdown of citrate in these organisms.

*Equilibrium between Citrate, Isocitrate and Cis-Aconitate.*—Martius (46) has shown that the conversion of citrate into  $\alpha$ -ketoglutarate is preceded by the rearrangement of citrate to isocitrate, *cis*-aconitate being an intermediate:



Martius (46) found that the three tricarboxylic acids are interconvertible in the presence of tissue extracts. They form an equilibrium when approximately 80% citric acid, 4% *cis*-aconitic acid and 16% isocitric acid are present (40°; pH 7.4) (Johnson (47)). These figures should be regarded as provisional; they will have to be revised when the analytical methods for the determination of the acids, especially of isocitric acid, have been perfected.

Isocitrate formed enzymically from citrate or *cis*-aconitate is optically active. Martius (46) isolated the compound in the form of the methyl ester of lacto-isocitric acid and found  $[\alpha]_D^{20}$  to be  $-61.50$  in dioxan. A similar value ( $-65.3^\circ$ ) was observed by Bruce (48) for the methyl ester of lacto-isocitric acid from blackberries—so far the only biological material where isocitric acid has been found to occur in quantities. The rotation increases much on addition of ammonium molybdate (as is the case with other  $\alpha$ -hydroxy acids),  $[\alpha]_D^{20}$  being  $+413$  when 5 ml. neutralized lacto-isocitric acid are mixed with 0.5 ml. glacial acetic acid and 4.5 ml. saturated ammonium molybdate.

Isocitric acid possesses two asymmetric carbon atoms and it should therefore exist in six different forms, four being optically active (*d,d*; *l,l*; *l,d*; *d,l*) and two being racemates (*d,d*/*l,l*; *d,l*/*l,d*). So far only one active form and one synthetic (49, 50) racemate

(which may be a mixture) have been isolated. Of the synthetic isocitric acid 50% are metabolized (46, 18). This is somewhat unexpected and may be explained by the assumption that either the enzymes can attack two active forms, or that the synthetic substance is not a mixture of the two racemates but contains only one of the two possible racemic forms. Martius (46) prefers the second alternative. In support of this view it can be pointed out that the instability of certain racemates has been shown for substances related to isocitric acid, *viz.*, ethylparaconic acids\* (51) where all six forms have been isolated; one pair of the racemic forms was found to be unstable and to be readily rearranged to the stable form.

*Aconitase.*—The name "aconitase" has been suggested for the enzyme, or enzymes, responsible for the interconversion of citric, aconitic and isocitric acids (52). The action of aconitase—the reversible hydration of a double bond—is analogous to that of fumarase though the configuration of the double bond differs in the two cases, being *cis*- in the former and *trans*- in the latter. Jacobsohn and Tapadinhas (53) report that some tissue extracts, for instance, from leaves of the medlar (*Mespilus germanica* L.), hydrate *cis*-aconitate but not fumarate; further that fluoride inhibits aconitase, but not fumarase, while mono-iodoacetate inhibits fumarase but not aconitase. These findings indicate that fumarase and aconitase are different enzymes.

Jacobsohn, *et al.* (54, 55), believe that the formation of isocitrate and of citrate from aconitate requires two different aconitases which they name  $\alpha$ - and  $\beta$ -aconitase according to the position of the hydroxy group formed through the agency of the enzyme (isocitric acid being considered as an  $\alpha$ -hydroxy and citric acid as a  $\beta$ -hydroxy acid). However, the evidence supporting this view, *i. e.*, certain alleged variations in the relative rates of two reactions, is not convincing. It has not been possible to separate the activity of the two enzymes.

Aconitase occurs in most, if not all animal tissues which are metabolically active. It has also been found in plants, *e. g.*, cucumber seeds, wheat and rye grains, peas, soya beans, and in bacteria (46, 53, 54, 55). It is absent from yeast.

*Enzymes Concerned with the Metabolism of Citrate.*—Aconitase appears to be the only enzyme activating citrate directly. As Martius (46) points out "citric dehydrogenase" is, in fact, isocitric dehydrogenase plus aconitase. Some properties of isocitric dehydrogenase have been described by Adler, *et al.* (19).

*Reactions of  $\alpha$ -Ketoglutarate.*—*Conversion into Succinate.*—The first experiments on the metabolism of  $\alpha$ -ketoglutarate were those of Neuberg and Ringer (56) who showed that fermenting yeast and "putrefying" bacteria convert the ketonic acid into succinic acid. The formation of

\* Lacto-isocitric acid is a carboxyparaconic acid.

succinate in animal tissues, though it had been postulated by earlier workers on the grounds of analogy with the metabolic behavior of other  $\alpha$ -ketonic acids, was first demonstrated by Krebs and Johnson (28). In muscle tissue succinate appears in large quantities when  $\alpha$ -ketoglutarate is oxidized in the presence of malonate.

*Dismutations of  $\alpha$ -Ketoglutarate.*—Two different anaerobic dismutations of  $\alpha$ -ketoglutarate occur in animal tissues. Weil-Malherbe (57) found the following reaction:



The rate of this reaction, as indicated by the increase in carbon dioxide production on addition of  $\alpha$ -ketoglutaric acid is fairly small as shown by the following figures (58):

Tissue	Without $\text{QCo}_2$	With 0.02 $M$ $\alpha$ -ketoglutarate
Sliced guinea pig kidney	2.0	3.1
Sliced rat kidney	2.4	3.4
Sliced rat liver	6.4	7.5
Sliced guinea pig brain	0.8	1.7
Sliced rat spleen	1.2	1.3
Minced pigeon breast muscle	0.1	1.6

In kidney cortex, and to a lesser extent in heart muscle  $\alpha$ -ketoglutarate may undergo another type of dismutation resulting in the synthesis of glutamic acid:



The following data indicate the rate of the reaction, as measured by the anaerobic carbon dioxide output:

Tissue	Substrate	$\text{QCo}_2$
Guinea pig kidney (sliced)	None	2.0
	0.02 $M$ $\alpha$ -ketoglutarate	3.1
	0.02 $M$ $\alpha$ -ketoglutarate; 0.02 $M$ $\text{NH}_4\text{Cl}$	7.9
Sheep heart (minced)	None	0.2
	0.02 $M$ $\alpha$ -ketoglutarate	0.7
	0.02 $M$ $\alpha$ -ketoglutarate; 0.02 $M$ $\text{NH}_4\text{Cl}$	2.1

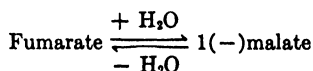
As compared with the oxygen uptake ( $\text{QO}_2$  for both tissues being  $-30$  to  $-40$  under similar conditions) the rate of reaction is low.

*Reactions of Succinate.*—*Formation of Fumarate.*—Batelli and Stern (59) discovered malate (which they believed to be optically inactive) as a product of the oxidation of succinate. Einbeck (60) showed later that fumarate is the primary product and that malate is formed by secondary hydration of the fumarate. Dakin (61) showed the



malic acid formed in muscle to be levorotatory. This is also true for the malic acid formed in other types of cells including microorganisms and plants.

**Reactions of Fumarate and 1(-)Malate.**—*Equilibrium between and 1(-)Malate.*—The tissues of higher animals, of many plants, and of many bacteria contain the enzyme fumarase which catalyzes the reversible reaction



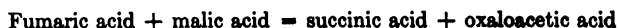
The enzyme has not been found in yeast (62). The interconversion of the two acids is usually very rapid compared with their formation or decomposition, at least in animal tissues (63), and the acids are therefore commonly encountered in their equilibrium mixture. The position of the equilibrium depends on the temperature (Jacobsen, 64). Krebs, Smyth and Evans, Jr. (65), found the following figures for the composition of the equilibrium mixture at pH 7.4.

Temperature	Ratio $\frac{\text{1(-)malate}}{\text{fumarate}}$
50° C.	2.65
40° C.	3.17
30° C.	3.54
20° C.	4.57

Data on the relative fumarase activity of various mammalian tissues are given by Breusch (63).

**Oxidation to Oxaloacetate.**—By analogy, *e. g.*, with  $\beta$ -hydroxybutyrate (Emden (66)) and crotonate (Friedmann (67)) it had long been expected that the biological oxidation of fumarate and malate would yield oxaloacetate. That this is actually the oxidation product was first demonstrated by Hahn and Haarmann (68) and later confirmed by Green (69). Szent-Györgyi (70), Green (69) and Laki (71) have discussed the question whether fumarate is oxidized directly. Differences in opinion have been settled and the consensus is now that fumarate is not oxidized as such, but only after conversion into malate.

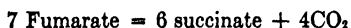
**Reduction to Succinate.**—Succinic dehydrogenase also catalyzes the hydrogenation of fumarate (Quastel and Whetham (72)). Suitable hydrogen donators are leucomethylene blue and dihydrocozymase. The reduction is observed in animal tissues when fumarate is added anaerobically, but the rate of this reaction is relatively slow, and the nature of the hydrogen donator is not clear in this case. Green's (69) experiments suggest that malate might be the hydrogen donator, according to the equation:



but as Green worked with artificially constructed enzyme systems his experiments only show that the above reaction *can* occur; to what extent it occurs in the tissue remains to be studied.

The following figures make it doubtful whether the reduction of fumarate is of major importance. When 267 mg. pigeon breast muscle, suspended in "phosphate saline" containing  $8 \times 10^{-6}$  moles of fumarate, were incubated anaerobically for 30 minutes,  $0.9 \times 10^{-7}$  moles of succinate were formed by reduction. The same quantity of muscle, when incubated aerobically with  $8 \times 10^{-6}$  moles of succinate, formed  $16 \times 10^{-7}$  moles of fumarate and malate. Thus the rate of the reduction of fumarate was less than 6% of the rate of the oxidation of succinate (18).

In certain bacteria, *e. g.*, *Escherichia coli* and *Aerobacter aerogenes*, the reduction of fumarate is much more rapid than in animal tissues (10). If fumarate is the only available substrate and if the conditions are anaerobic one molecule of fumarate undergoes complete oxidation and serves as a hydrogen donator for 6 other molecules:



If other oxidizable substances are present, *e. g.*, glucose, lactic acid, glycerol or acetate, these can act as hydrogen donators (10). Similar reactions have been found to occur in propionic acid bacteria (73). The rate of the anaerobic reduction of fumarate in the organisms mentioned is of the same order of magnitude as the rate of oxidation of succinate by molecular oxygen.

**Reactions of Oxaloacetate.**—*Decarboxylation.*—Like all  $\beta$ -ketonic acids, oxaloacetic acid is unstable in aqueous solution; it undergoes the "ketone decomposition" into  $\text{CO}_2$  and pyruvic acid:



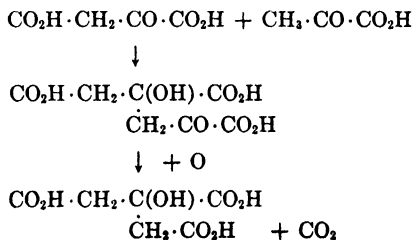
at pH 4 and  $20^\circ$  the rate of the ketone decomposition varies between 6 and 18% per hour (74). It is lower in neutral solutions. The reaction is catalyzed by amino compounds, including amino acids, proteins, aniline, and by multivalent inorganic ions (Mg, Cu, Al) (74). Tissue suspensions also catalyze the decomposition (75, 76, 77, 78, 29) but as boiled tissue has much the same effect as fresh material, it is very doubtful that the reaction is due to a specific enzyme. There can be no doubt, however, that *Micrococcus leisodeikticus* possesses a heat labile enzyme system which catalyzes the decarboxylation of oxaloacetate (Krampitz and Werkman (79); (18)). Magnesium ions are a component part of this system, whereas cocarboxylase is not.

*Reduction to Malate and Fumarate.*—Szent-Györgyi and his team (70, 80) discovered in 1935 that suspensions of pigeon breast muscle and other tissues are capable of reducing added oxaloacetate with great rapidity, the main reduction product being a mixture of malate and fumarate. For instance, 0.4 g. muscle suspended in 4 ml. medium was found to reduce more than 4 mg. oxaloacetate in 5 minutes. The rate of reaction is somewhat lower in other tissues (29, 81). *Escherichia coli* (10), propionic acid bacteria (73) and many other bacteria (18) also reduce oxaloacetate.

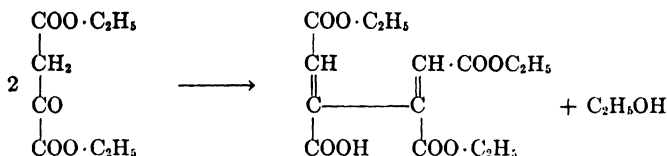
Where fumarate can be reduced to succinate, as in *Escherichia coli* or propionic acid bacteria, succinate is the main end product of the reduction. In animal tissues the reduction of fumarate is very slow as compared with its formation from oxaloacetate and the chief product is, therefore, the equilibrium mixture of fumarate and malate.

*Formation of Citrate and Related Substances.*—When oxaloacetate is added to muscle suspensions, decarboxylation and reduction never account for the total oxaloacetate which disappears (80, 29). Under conditions where the nonenzymic decarboxylation is small, the yield of malate and fumarate does not exceed 75%; it is generally of the order of 50% (81). The remaining fraction can be accounted for largely as  $\alpha$ -ketoglutarate and citrate, the yield of the former being 10–25%, that of the latter, 1–2% (28, 81). The yield of citrate depends among other factors on the concentration of oxaloacetate. Only at fairly high concentrations (0.05–0.1 *M*) is citrate found in appreciable quantities. The yields of  $\alpha$ -ketoglutarate, on the other hand, are almost independent of the concentrations of added oxaloacetate. The simultaneous formation of citrate and  $\alpha$ -ketoglutarate may not be unexpected in view of the ready conversion of the former into the latter.

With regard to the mechanism of the formation of citrate, it is of interest to record two test tube experiments with oxaloacetate. Knoop and Martius (82) have shown that a mixture of oxaloacetate and pyruvate, dissolved in aqueous sodium carbonate, forms citrate when treated with hydrogen peroxide. The primary step in this reaction is in all probability an aldol condensation and this is followed by oxidative decarboxylation.

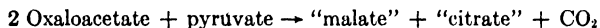


Claisen and Hori (83) found that 2 molecules of ethyl oxaloacetate condense in the presence of potassium acetate to form the triethyl ester of oxaloaconitic acid:

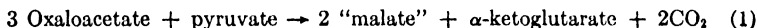


While in the first case the condensation leads to a hydroxy acid (citric acid) the reaction product is an unsaturated compound (an aconitic acid derivative) in the second.

*"Fermentation" of Oxaloacetate.*—The anaerobic reactions of oxaloacetate in animal tissues and in bacteria may be regarded as fermentations, *i. e.*, coupled oxido-reductions occurring in the absence of molecular oxygen. The reductive formation of malate and fumarate is coupled with the oxidative formation of citrate and  $\alpha$ -ketoglutarate. The two stages of the "fermentation" may therefore be formulated thus:

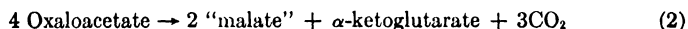


The over-all effect of the two reactions is:



In these schemes "malate" stands for the system: fumarate  $\rightleftharpoons$  1(-) malate and "citrate" for the system citrate  $\rightleftharpoons$  *cis*-aconitate  $\rightleftharpoons$  isocitrate.

Experimentally the pyruvate taking part in reaction (1) will usually, though not always, arise by decarboxylation of oxaloacetate. In this case reaction (1) becomes:



Data on pigeon breast muscle agree fairly well with these schemes (1) (2) (81). Complete agreement cannot be expected, owing to various side reactions such as the reduction of fumarate to succinate, the oxidation of  $\alpha$ -ketoglutarate to succinate and the formation of pyruvate from carbohydrate derivatives (81).

The "fermentation" of oxaloacetate in bacteria is somewhat different in detail though the underlying principle is the same. The common principle is the reduction of one fraction of the added oxaloacetate coupled with the oxidation of another fraction. The difference is in the nature of the oxidative and reductive reactions. In animal tissues the reduction leads

mainly to "malic acid." In *Escherichia coli* and in propionic acid bacteria it proceeds further to the stage of succinate. The details of the oxidative process in *Escherichia coli* are not yet clear. The main end product is carbon dioxide. In propionic acid bacteria the oxidative equivalent is, among others, the reaction pyruvate = acetate + CO<sub>2</sub>.

**Reactions of Pyruvate.**—*Rate of Removal.*—In most animal tissues, and in many other living cells, added pyruvate causes an increase in the oxygen uptake and a removal of the pyruvate.

Data on the rate of the oxidative decomposition of pyruvic acid in various animal tissues are given in the following table.

RATE OF REMOVAL OF PYRUVATE BY ANIMAL TISSUES (8, 11, 28, 84, 85) (40°)

Tissue	Q <sub>pyruvate</sub>
Rat brain (sliced) *	-9
Rat liver (sliced)	-9
Rat kidney cortex (sliced)	-23
Rat spleen (sliced)	-5
Rat testis	-14
Rat intestine	-3
Pigeon breast muscle (minced)	-15
Sheep's heart (minced)	-8
Pigeon liver (minced)	-45

Pyruvate also disappears under anaerobic conditions, but generally at a considerably lower rate.

*Factors Affecting the Oxidation of Pyruvate in Muscle.*—In pigeon breast muscle (8) or in mammalian heart muscle (Smyth (11)) malonate and fumarate (or malate) have characteristic effects on the rate of the oxidative removal of pyruvate.

Malonate inhibits the oxidation. The inhibition is, for instance, 77% at 0.001 *M* malonate, and 92% at 0.025 *M* malonate (8). Most, if not all, of the removal of pyruvate remaining in the presence of 0.025 *M* malonate is due to anaerobic reactions which are not appreciably inhibited by malonate.

Fumarate abolishes the inhibitory effects of malonate. The extent of the fumarate effect depends on the relative concentrations of malonate and of fumarate: if the malonate concentration is low (0.001 *M*) the inhibitory effect is completely removed by 0.0025 *M* fumarate; if the malonate concentration is high (0.025 *M*) fumarate restores the oxidative removal of only an equivalent quantity of pyruvate. In the absence of malonate,

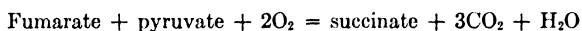
fumarate has only a slight effect on the oxidation of pyruvate. These facts are illustrated by the following data (8):

OXIDATIVE REMOVAL OF PYRUVATE BY PIGEON BREAST MUSCLE (8)

(4 Ml. muscle suspension containing 267 mg. fresh muscle; 0.02 *M* pyruvate.)

	Substances added	Absolute quantity of added fumarate, $\mu$ l.	$\mu$ l. pyruvate used		$\mu$ l. extra pyruvate used on addition of fumarate
			60 min.	180 min.	
Expt. I	None	...	556	793	...
	0.0025 <i>M</i> fumarate	224	672	928	...
	0.001 <i>M</i> malonate	...	40	184	...
	0.001 <i>M</i> malonate; 0.0025 <i>M</i> fumarate	224	666	928	744
Expt. II	None	...	868	1046	...
	0.025 <i>M</i> malonate	...	76	92	...
	0.025 <i>M</i> fumarate	224	740	1005	...
	0.025 <i>M</i> malonate; 0.025 <i>M</i> fumarate	224	244	313	221

*Incomplete Oxidation in Muscle in the Presence of Malonate.*—When no inhibitor is added, the oxidation of pyruvate in minced muscle is usually complete. Per molecule of pyruvate  $2\frac{1}{2}$  molecules of  $O_2$  are absorbed and 3 molecules of  $CO_2$  are formed. In the presence of malonate and fumarate the rate of pyruvate removal may be "normal," but the products of oxidation are different. The oxidation is incomplete. Per molecule of pyruvate and fumarate present, approximately 2 molecules of  $O_2$  are absorbed and 3 molecules of  $CO_2$  are formed ( $5\frac{1}{2}$  molecules of  $O_2$  and 7 molecules of  $CO_2$  are calculated for the complete oxidation of the two substrates). As a product of incomplete oxidation succinate appears in the solution in quantities approximately equivalent to the amounts of pyruvate and fumarate used. The data agree roughly with the equation:



According to this equation the amounts of fumarate, pyruvate and succinate should be equivalent. Experimentally (8) the following proportions were found 1:1.35:0.99; 1:1.19:0.90; 1:0.98:0.81. Such deviations from the above equation can easily be explained by side reactions (8).

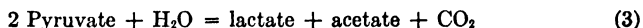
*Formation of Citrate and  $\alpha$ -Ketoglutarate in Muscle.*—Fumarate yields small quantities of citrate and  $\alpha$ -ketoglutarate on addition to respiring muscle suspensions. The amounts of both substances are much increased when pyruvate and fumarate are added together, while pyruvate alone

yields no significant quantities of the two substances. In terms of the added fumarate the yields of  $\alpha$ -ketoglutarate reach 50% and those of citrate 15% (8).

*Oxidation to Acetate.*—The formation of acetate from pyruvate has long been known to take place in bacteria. In animal tissues acetate was identified as an oxidation product of pyruvate by Krebs and Johnson (28), by Weil-Malherbe (57) and by Long (86).

*Reduction to Lactate.*—The reduction to lactate is regarded as the final step in the formation of lactate from carbohydrate. The hydrogen donator is in this case a triose phosphate (diphosphoglyceraldehyde), cozymase acting as a hydrogen carrier.

*Dismutations.*—Krebs and Johnson (28) and independently Lipmann (87) and Weil-Malherbe (57) found that pyruvate dismutates anaerobically in animal tissues according to the following equation:



The rate of this reaction varies from tissue to tissue. It can be conveniently measured by the determination of the extra carbon dioxide formed on addition of pyruvate under anaerobic conditions. Examples (28) are given in the following table.

Tissue	QCO <sub>2</sub>	
	Without pyruvate	With 0.02 M pyruvate
Rat liver (sliced; first 40 min.)	4.8	13.4
Rat liver (sliced; second 40 min.)	1.5	3.1
Pigeon liver (sliced)	4.0	7.9
Rat kidney (sliced)	2.1	5.9
Rat brain cortex (sliced)	0.4	3.3
Rat testis	1.0	5.0
Pigeon breast muscle (minced)	0.3	1.8
Sheep heart (minced)	0.1	1.3

It is noteworthy that the reaction is comparatively slow in minced muscle where the aerobic oxidation is very rapid.

The dismutation of pyruvate also occurs in *Staphylococcus aureus* and *albus*, *Streptococcus faecalis* and in the gonococcus (88, 89), furthermore in *Lactobacillus delbrueckii* (90) and under some conditions—at low pH—in *Escherichia coli* (21, 91).

A second type of dismutation—an intramolecular dismutation—occurs in *Escherichia coli* (21), *Streptococcus haemolyticus* (89), *Aerobacter aero-*

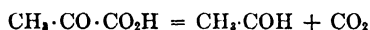
*genes* (18) and *Proteus vulgaris* (18), resulting in formation of acetate and formate:



Although this is a cleavage reaction in which the elements of water are taken up, it is not a hydrolysis, any more than reaction (3) is a hydrolysis. Both (3) and (4) are oxido-reductions, or "fermentations" of pyruvate which yield energy.

Krebs and Johnson (28) (see also Weil-Malherbe (57) and Green, *et al.* (92)) have presented evidence which indicates that in animal tissues either one or both molecules of pyruvate in (3) can be replaced by other  $\alpha$ - or  $\beta$ -ketonic acids, *e. g.*,  $\alpha$ -ketoglutarate, acetoacetate and oxaloacetate.

*Decarboxylation.*—Yeast extracts—though not intact yeast cells—convert pyruvate anaerobically into acetaldehyde and  $\text{CO}_2$ :



Green, *et al.* (93), observed the same reaction with a purified enzyme obtained from sheep heart. This presents a puzzle, for cardiac or skeletal muscle, unlike yeast, seems to be unable to metabolize added acetaldehyde in appreciable quantities. This has been taken to indicate that acetaldehyde is not a major intermediate in muscle metabolism. It is not impossible that the enzyme of Green, *et al.*, has undergone a modification during the purifying process and is thus an artifact. Further work is required to elucidate the matter.

*Synthesis of Acetoacetic and  $\beta$ -Hydroxybutyric Acids.*—Annau (94) and Edson (95) have shown that liver forms acetoacetate on addition of pyruvate. The yields are considerably increased when ammonium chloride (94, 95) or malonate (94, 96, 97) are also present, as seen in the following table.

FORMATION OF ACETOACETATE FROM PYRUVATE (95, 97)

Tissue	Substrates added	Acetoacetate
Rat liver (well nourished, sliced)	None	0.22
Rat liver (well nourished, sliced)	0.01 <i>M</i> pyruvate	0.44
Rat liver (well nourished, sliced)	0.01 <i>M</i> pyruvate; 0.04 <i>M</i> $\text{NH}_4\text{Cl}$	1.96
Rat liver (well nourished, sliced)	0.04 <i>M</i> $\text{NH}_4\text{Cl}$	0.77
Pigeon liver (minced)	0.03 <i>M</i> pyruvate	0.95
Pigeon liver (minced)	0.03 <i>M</i> pyruvate; 0.025 <i>M</i> malonate	5.8



The formation of acetoacetate is generally accompanied by a formation of some  $\beta$ -hydroxybutyrate. The methods available for the determination of small amounts of  $\beta$ -hydroxybutyrate, however, are not specific and the data presented by various authors (28, 95, 96), therefore, contain an element of uncertainty. The formation of acetoacetate from pyruvate has so far been demonstrated in liver only. Krebs and Johnson (28) observed a small " $\beta$ -hydroxybutyrate" formation in muscle and other tissues on anaerobic incubation with pyruvate which, as just pointed out, requires confirmation by independent methods.

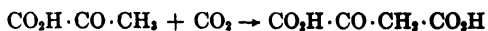
*Synthesis of Glutamine and  $\alpha$ -Ketoglutaric Acid.*—Örström, Örström, Krebs and Eggleston (98) found that avian liver is capable of synthesizing glutamine from ammonium pyruvate and expressed the view that the synthesis of  $\alpha$ -ketoglutarate is probably an intermediary step in this reaction. Evans (97) showed that  $\alpha$ -ketoglutarate is, in fact, formed in considerable quantities when pigeon liver is incubated with sodium pyruvate.  $Q_{\text{ketoglutarate}}$  was, for instance, 8.5, while  $Q_{\text{glutamine}}$  in the experiments of Örström, *et al.*, was 3–5.

This synthesis of  $\alpha$ -ketoglutarate differs from that taking place in muscle in that it occurs without the addition of a four-carbon dicarboxylic acid. In muscle  $\alpha$ -ketoglutarate is not formed in quantities unless pyruvate plus fumarate, or oxaloacetate, are added.

*Carboxylation.*—The synthesis of  $\alpha$ -ketoglutarate in pigeon liver is accompanied by the formation of considerable quantities of malate and fumarate and smaller quantities of citrate and succinate (Krebs and Eggleston (84)). The simultaneous appearance of these substances might at first be taken as a matter of course, since they are inconvertible, if the conditions are aerobic. However, the substances also arise from pyruvate under anaerobic conditions when an oxidative conversion of  $\alpha$ -ketoglutarate into fumarate is impossible. Moreover, even aerobically the total absorption of oxygen was found to be too small—under certain conditions—to account for a primary synthesis of  $\alpha$ -ketoglutarate, and subsequent oxidation to succinate, fumarate, malate and citrate.

The substances arising from pyruvate in pigeon liver are the same as those formed on addition of oxaloacetate. The proportions in which the substances appear are similar with oxaloacetate and with pyruvate, while the rate is more rapid with oxaloacetate (Table IV).

The simplest explanation for the fact that pyruvate and oxaloacetate yield the same products is the assumption that pyruvate is first converted into oxaloacetate by combining with carbon dioxide:



(5)

To prove the occurrence of this reaction it would be ideal to demonstrate the removal of pyruvate and carbon dioxide and the formation of stoichiometric quantities of oxaloacetate. As it happens, however, oxaloacetate is one of the most reactive substances among intermediary metabolites in animal tissues; 1 mg. (dry weight) of pigeon liver can metabolize 0.011 mg. oxaloacetate in 1 minute (not counting the decomposition to pyruvate and carbon dioxide (18)). In pigeon breast muscle the rate of reaction is of the same order (80). This is about ten times as much oxaloacetate as may be expected, on account of the yields of  $\alpha$ -ketoglutarate, fumarate and malate, to arise under similar conditions, from reaction (5). Hence the prospects of a successful isolation of the oxaloacetate formed by reaction (5) are very slight, if not nil.

TABLE IV

## ANAEROBIC METABOLISM OF PYRUVATE AND OXALOACETATE IN PIGEON LIVER

Substrate:	Pyruvate	Oxaloacetate
Substrate used	890 $\mu$ l.	1344 $\mu$ l.
Metabolites formed		
Succinate	45 $\mu$ l. (yield 5.1%)	61 $\mu$ l. (yield 4.5%)
$\alpha$ -Ketoglutarate	131 $\mu$ l. (yield 14.7%)	197 $\mu$ l. (yield 14.6%)
Fumarate + malate	334 $\mu$ l. (yield 37.6%)	580 $\mu$ l. (yield 43.1%)
Citrate	12 $\mu$ l. (yield 1.3%)	11 $\mu$ l. (yield 0.8%)

The data for pyruvate are taken from Krebs and Eggleston (84), those for oxaloacetate from Evans (97). The yields are expressed as percentage of substrate used. Period of incubation: 60 minutes in the pyruvate experiment, 40 minutes in the oxaloacetate experiment. The figures refer to 4 ml. liver suspensions containing 400 mg. tissue (wet weight).

There remains the alternative method of identifying oxaloacetate from its derivatives. "Derivatives" of oxaloacetate, in this connection, are the substances which arise from oxaloacetate in the anaerobic fermentation, viz.,  $\alpha$ -ketoglutarate, fumarate, malate, citrate and succinate. While the formation of one or two of the substances may not be specific for oxaloacetate, the complete combination of the five substances in definite proportions, as shown in Table IV, must be regarded as specific for oxaloacetate, for no other substance (except pyruvate) is known to give this combination anaerobically. In the view of the writer, the conclusion that pyruvate is converted into oxaloacetate according to reaction (5) is therefore inescapable. This conclusion is further supported by the observation that the rate of pyruvate removal, and of  $\alpha$ -ketoglutarate formation, is dependent on the concentration of bicarbonate and carbon dioxide (84).

The direct participation of carbon dioxide in the synthesis of  $\alpha$ -ketoglutarate was demonstrated with the help of carbon isotopes by Evans and Slotin (99) and later by Wood, Werkman, Hemingway and Nier (100). The fixed carbon was found to be present in the carboxyl group adjacent to the carbonyl. The second carboxyl of  $\alpha$ -ketoglutarate contained no measurable quantities of the isotope. The location of the fixed carbon is a matter of great importance, for it allows conclusions to be made with regard to the mechanism of the formation of  $\alpha$ -ketoglutarate. This is discussed later. Wood, *et al.* (101), also showed that the malate fumarate and succinate formed anaerobically from pyruvate, as expected, contain fixed carbon in their carboxyl groups.

Already before carbon isotope experiments on animal tissues or bacteria were published, Krebs and Eggleston (84) came to the conclusion "that the force of the cumulative evidence leaves little doubt about the occurrence of reaction (5)." Such doubts as then existed were due to the entirely novel feature of an "assimilation" of carbon dioxide in animal tissues. The tracer experiments have since established carbon dioxide assimilation beyond doubt and the writer therefore regards the evidence for the occurrence of reaction (5) in pigeon liver as complete.

The available evidence indicates that carboxylation does not occur in muscle, at least not at a rate comparable with that in liver, for muscle, unlike liver, does not synthesize significant quantities of  $\alpha$ -ketoglutarate from pyruvate. On the other hand, brain and guinea pig kidney cortex like pigeon liver appear to synthesize (though at a slow rate) glutamine from ammonium pyruvate (104, 18) and on the grounds of analogy it is probable that this involves the carboxylation of pyruvate.

As regards the occurrence of carboxylation in bacteria and molds, the reader is referred to the review of Werkman and Wood (103).

**Reactions of Acetate.**—*Oxidative Removal.*—When acetate is added to slices of kidney cortex, the oxygen uptake increases and the concentration of the bicarbonate of the medium gradually rises (85, 105). This increase in the bicarbonate concentrations indicates a removal of the added acid, either by complete oxidation or by conversion into a neutral substance. The highest rates of acetate removal, as measured by the formation of bicarbonate (more specific micro methods are lacking), has been found in guinea pig kidney cortex where  $Q_{\text{bicarbonate}}$  is 7 to 8. Brain and liver of guinea pig give a  $Q_{\text{bicarbonate}}$  value between 1 and 2 (105). An oxidative removal of acetate in perfused muscle (with the formation of formate) has been reported by Toeniessen and Brinkmann (106) but the evidence is hardly convincing. The published data only indicate a removal of acetate from the perfusion liquid and do not differentiate between acetate deposited in the tissues and acetate oxidized.

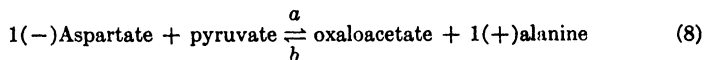
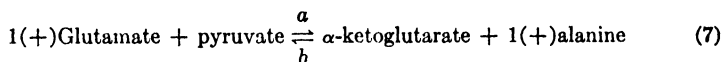
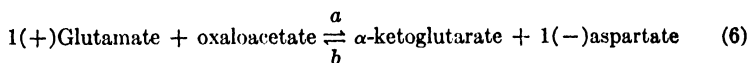
*Formation of Acetoacetate and  $\beta$ -Hydroxybutyrate.*—Acetoacetate and  $\beta$ -hydroxybutyrate arise on addition of acetate to liver tissue (95, 107). The effect is greatest in the liver of starved animals. It is doubtful whether the "ketone bodies" are formed from acetate in any other tissues.

*Reactions of Acetoacetate and  $\beta$ -Hydroxybutyrate.*—*Oxidative Removal.*—While the oxidation of fatty acids in the animal body occurs in a few tissues only (chiefly in liver, to a lesser extent in kidney cortex and in very small quantities in spleen and testis (108)), two oxidation products of fatty acids—acetoacetate and 1(-) $\beta$ -hydroxybutyrate—are metabolized in the majority of tissues, including muscle (108, 109). The oxidative decomposition of the "ketone bodies" has been widely studied (24, 110, 111, 112, 113, 114) but no intermediary oxidation products have as yet been identified. Acetone is not regarded as a normal intermediate; it is formed through a nonenzymic side reaction.

*Interconversion.*—Acetoacetate and  $\beta$ -hydroxybutyrate are readily interconvertible in most animal tissues (111, 109). The reduction of the ketonic to the hydroxy acid is observed when acetoacetate is added to tissues anaerobically while the reverse reaction prevails when  $\beta$ -hydroxybutyrate is added aerobically.

The oxidative processes with which the anaerobic reduction of acetoacetate is necessarily coupled are but incompletely known. In some instances the oxidative decarboxylation of  $\alpha$ -ketonic acids was involved (see 28).

*Transaminations.*—The three ketonic acids which arise in the course of the oxidation of citrate— $\alpha$ -ketoglutarate, oxaloacetate and pyruvate—can take part in further reactions, not yet discussed, in which the ketonic acids are converted into, or formed from, their corresponding amino acids. The following three reversible reactions occur:



This type of change, now called "transamination," was discovered by Braunstein and Kritzmann (115) and studied by Cohen (116).

Cohen (116) gives the following rates of the various reactions for animal tissues:

Tissue	Qtransamination	$\frac{\mu\text{l. substrate transaminated}}{\text{mg. dry tissue} \times \text{hours}}$	
	Reaction (6a)	Reaction (7a)	Reaction (8a)
Heart (rat)	425	7	7
Skeletal muscle (rat)	316	13	1
Pigeon breast muscle	400	40	..
Brain (rat)	260	2	8
Brain (cat)	210	..	..
Liver (rat)	245	46	10
Liver (cat foetus)	64	..	..
Kidney (rat)	245	3	3
Testis (rat)	150	..	..
Lung (rat)	51	..	..
Spleen (rat)	16	..	..
Sarcoma S.37	57	..	..

Thus the rate of (6a) is very rapid, approximately ten times as high as is the maximal rate of the  $O_2$  uptake. The rates of (7a) and (8b) are very low in most tissues. Only liver and pigeon breast muscle show an appreciable rate in the case of (7a).

**Catalytic Effects.**—It is a common feature of all the reactions so far described that the added metabolite disappears in stoichiometric proportions during the reaction. Under some conditions, especially when added in small quantities, the substances of the "citric acid series" (Table I) have effects on the metabolism which are not in stoichiometric proportion to the added substrate and are not necessarily accompanied by the disappearance of the added substrates. The first to discover such "catalytic" effects was Szent-Györgyi (70, 80) who found that succinate, fumarate, malate or oxaloacetate catalytically increases the oxygen uptake and carbon dioxide production of muscle and of other animal tissues. Stare and Baumann (6) confirmed these findings; on addition of fumarate to pigeon breast muscle suspensions, for instance, an extra oxygen uptake was measured which was five times the quantity required for the complete oxidation of the added substrate. Krebs and Johnson (42) found similar effects with citrate.

The same substances catalyze the oxidative removal of pyruvate in skeletal and cardiac muscle (8, 11).

Fumarate and oxaloacetate furthermore catalyze a number of anaerobic fermentations in microorganisms: the fermentation of pyruvate in *Escherichia coli* (21), the dismutation of pyruvate in *Staphylococcus* (117), the fermentation of glycerol and other substances in propionic acid bacteria (73), the fermentation of citrate in *Aerobacter aerogenes* (18).

**Summary.**—Table V is a summary of the chemical transformations discussed in the preceding pages. The table includes the major reactions which at present are known to occur in animal tissues burning essentially carbohydrate and which, therefore, may be expected to be associated with carbohydrate oxidation. These reactions form the chief factual foundation for all theoretical considerations on the mechanism of carbohydrate oxidation. On the whole, the opinions of various authors appear to be in agreement with regard to these facts.

As the table is intended to present experimental observations all details involving hypothetical considerations, such as stoichiometric relations, and intermediary mechanisms, obviously highly complex in some cases, are omitted at the present stage.

## II. Reaction Schemes (Theory of Carbohydrate Oxidation)

### 1. *General Principles Concerning the Theory of Carbohydrate Oxidation*

**Object and Scope of the Theory.**—The chief aim of the theory of carbohydrate oxidation is to describe, step by step, the chemical changes of the carbohydrate molecule leading to the formation of carbon dioxide and water. At the same time when applied to muscle the theory should account for the specific enzymic equipment of this tissue which enables it to carry out the reactions listed in Table V. This follows from the principle discussed at the beginning of this article (Section I, 1).

Attempts have been made from time to time to formulate a theory complying with these postulates. The first attempt was that of Thunberg (4), the latest is a modified citric acid cycle. The merits or demerits of such schemes have recently been widely discussed, but in the view of the writer a certain amount of the discussion has sprung from misconceptions about the object and the scope of the schemes. The following remarks are therefore offered as an effort to clarify the position.

The schemes under consideration are of the nature of hypotheses. Like all hypotheses, they are suppositions which are made in order to account for experimental observations and to serve as a starting point for further investigations. For reasons to be stated later they belong to those types of hypotheses which are destined always to remain hypotheses. They can never become facts, *i. e.*, something which can be observed, and therefore they cannot be proved (if to prove means to establish as a fact)—though they can be disproved. It is therefore beside the point if it is stated that such and such a scheme has not been “proved.” The value of a reaction scheme should be assessed on the grounds of the observations

TABLE V  
SUMMARY OF THE METABOLIC ACTIVITIES OBSERVED IN ANIMAL TISSUES ASSOCIATED WITH CARBOHYDRATE METABOLISM†

I Complete oxidations	II Incomplete oxidations or Nonoxidative processes	III Catalytic effects
Carbohydrate Citrate <i>cis</i> -Aconitate Isocitrate $\alpha$ -Ketoglutarate Succinate Fumarate Malate Oxaloacetate Pyruvate $\alpha$ -Hydroxyglutarate Glutamate Aspartate Alanine	$+ O_2 \rightarrow CO_2 + H_2O$ Carbohydrate + O <sub>2</sub> Citrate $\rightarrow CO_2 + H_2O$ <i>cis</i> -Aconitate $\rightleftharpoons$ <i>cis</i> -aconitate Isocitrate $\rightleftharpoons$ isocitrate $\alpha$ -Ketoglutarate $\rightarrow \alpha$ -ketoglutarate + CO <sub>2</sub> * $\alpha$ -Ketoglutarate $\rightleftharpoons$ succinate + CO <sub>2</sub> $\alpha$ -Ketoglutarate $\rightleftharpoons$ $\alpha$ -hydroxyglutarate Succinate $\rightleftharpoons$ glutamate Fumarate $\rightleftharpoons$ fumarate Malate $\rightleftharpoons$ malate Oxaloacetate $\rightleftharpoons$ oxaloacetate Oxaloacetate $\rightarrow$ pyruvate + CO <sub>2</sub> Oxaloacetate $\rightleftharpoons$ aspartate Oxaloacetate $\rightarrow$ citrate Oxaloacetate $\rightarrow \alpha$ -ketoglutarate *Pyruvate $\rightarrow$ acetate + CO <sub>2</sub> Pyruvate $\rightleftharpoons$ lactate **Pyruvate + CO <sub>2</sub> $\rightarrow$ oxaloacetate *Pyruvate $\rightarrow$ acetaldehyde + CO <sub>2</sub> Pyruvate + fumarate $\rightarrow$ citrate Pyruvate + fumarate $\rightarrow \alpha$ -ketoglutarate Pyruvate $\rightarrow$ acetoacetate **Pyruvate $\rightarrow \alpha$ -ketoglutarate **Pyruvate + NH <sub>3</sub> $\rightarrow$ glutamine Pyruvate $\rightleftharpoons$ alanine **Acetate $\rightarrow$ acetoacetate *Acetoacetate $\rightleftharpoons$ $\alpha$ -hydroxybutyrate	Citrate, succinate, fumarate, malate or oxaloacetate catalyze O <sub>2</sub> uptake and CO <sub>2</sub> production, and oxidation of pyruvate

† The reactions known to take part in the anaerobic glycolysis are omitted from the table. They are indicated by the arrows in Table I, column I.

\* The reactions marked with one asterisk are comparatively slow.

\*\* The reactions marked with two asterisks have not been observed in muscle.

which it "explains" and the discoveries to which it has given rise when used as a working hypothesis; if a scheme does not explain every observation, this should not be weighed too heavily against the scheme. For an imperfect hypothesis which accounts for some facts and can guide further research is better than no hypothesis at all. Moreover, the inability of a scheme to account for a certain observation may not be due to the incorrectness, but rather to the incompleteness of the scheme as will be shown in the following section.

**Incompleteness of the Theory.**—Knowledge of the chemical organization of living matter is so scanty that one cannot expect to elaborate a complete, if hypothetical, picture of any complex metabolic process. Hence the theory of carbohydrate oxidation must necessarily remain incomplete. This is often overlooked when deductions are made from the theory and when these are found to clash with experimental findings. For instance, according to the theory of the citric acid cycle, added citrate is expected to restore the oxygen consumption of the malonate poisoned muscle at least as effectively as does  $\alpha$ -ketoglutarate. The experiment shows that under certain conditions this is not the case (31) and the conclusion has been drawn that it is doubtful whether citrate represents an essential stage in the respiration of pigeon breast muscle (31). In fact, the theory makes no detailed statement about the behavior of *added* citrate; it only states that citrate is an intermediate. It is true that, therefore, one might reasonably expect that *added* citrate is rapidly metabolized, but this is by no means necessarily the case. Addition of citrate results in a much higher concentration than does the intermediary formation of citrate. This higher concentration may cause inhibitions; either by combining with other enzymes or by combining with ions (32) required for oxidations. Inhibitions have actually been found (see Section II, also (118)).

Another example of the "incompleteness" of current reaction schemes may be drawn from the theory of the alcoholic fermentation. The generally accepted scheme includes the reaction  $\text{pyruvic acid} \rightarrow \text{CO}_2 + \text{acetaldehyde}$ . One would therefore expect this reaction to occur when pyruvate is added anaerobically to a suspension of yeast cells. In fact, however, added pyruvate is not anaerobically metabolized at pH 5.6 or 7.0, although it is rapidly oxidized in air (Smythe (119)). No one has seriously suggested that this shows that the scheme of Embden, Meyerhof and Neuberg is incorrect. Smythe thinks that the non-reactivity of pyruvate may be due to a nonpermeability of the yeast cell under anaerobic conditions.

No reaction scheme can be expected to be free from discrepancies of the type just described. As already stated such discrepancies usually indicate that the schemes are incomplete rather than incorrect.



**Significance of the Experimental Findings.**—Opinions sometimes seem to differ on the significance which should be attached to the occurrence of a chemical reaction observed in an experiment on biological material. For the purpose of the following discussion the writer has adopted, as a guiding principle, Hopkins' remark, already quoted, "The body . . . is in general able to deal only with what is customary to it" (1). It is therefore assumed that any rapid metabolic reaction found in muscle tissue is likely to be associated, directly or indirectly, with carbohydrate metabolism. The argument is, of course, not conclusive and has occasionally led astray. Its force is the greater, the more limited the metabolic activity of the material in question. It can hardly be applied to experiments on liver or kidney, where fatty acids, amino acids and carbohydrate are oxidized, or to those bacteria which metabolize many substrates, *e. g.*, *Escherichia coli* and *Aerobacter aerogenes*. It may be applicable, on the other hand, to propionic acid bacteria, whose metabolic capacity, especially in "resting" cells and under anaerobic conditions, is confined to a comparatively small set of reactions. If it is shown, for instance, that these organisms are capable of rapidly reducing oxaloacetate to succinate, this is, in the view of the writer, powerful evidence in support of the view that this reaction plays a part in the "normal" formation of succinate.

**What Can Be Proved?**—Experiments can "prove," *i. e.*, establish as a fact, the occurrence of certain chemical changes under a given set of conditions. In this sense the reactions listed in Table V have in the view of the writer been proved to occur. But the proof applies to the experimental conditions only and these are always more or less "unphysiological," as it is not possible to investigate intermediary reactions under "physiological" conditions. Physiologically most intermediates exist only transitorily, *i. e.*, in minute quantities. Moreover, they only occur intracellularly. These circumstances preclude their identification under "physiological" conditions. To investigate intermediary metabolism, the concentration of the metabolite must be artificially raised, or poisons must be added, and/or the tissue has to be removed from its normal site and to be perfused, or sliced, or minced, or extracted. The statement, therefore, that the evidence is valid for living tissues under "physiological" conditions always implies the assumption that the reactions occur under conditions different from those of the experiment. As far as one can see, this state of affairs is bound to persist, and for this reason the theory of intermediary reaction mechanism is bound always to remain a theory.

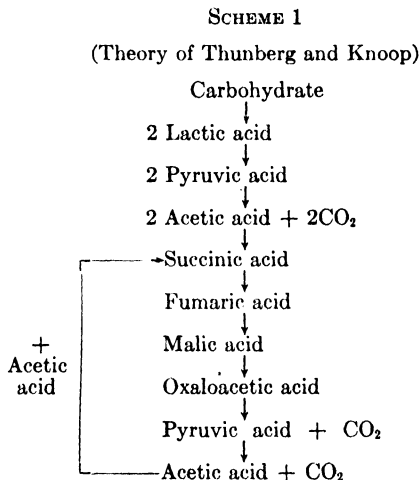
In short, while it can be proved that a tissue or a cell has the ability to perform certain reactions, the "physiological" occurrence of the reactions must remain an assumption. If one agrees with Hopkins' contention that, in general, a tissue is able to deal only with what is customary to it, the demonstration of an intermediary reaction under experimental conditions can be regarded as powerful evidence, though not a final proof, that the reaction is part of the normal metabolism of the tissue.

This is equally true for animal tissues and for microorganisms. Although many properties of microorganisms can be studied in the "physiological" medium the analysis

of the intermediary metabolism necessitates interference with intracellular mechanism. This can only be achieved by breaking up the cells, or by addition of "unphysiological" chemicals which inhibit catalysts or alter permeability. The history of the elucidation of the alcoholic fermentation in yeast illustrates this point, for all major discoveries in this field were made, and probably *could* only be made, either on press juice (Büchner) or maceration juice (Lebedev) or on cells treated with poisons such as toluene, chloroform, acetone or fluoride.

## 2. Earlier Schemes of Carbohydrate Oxidation

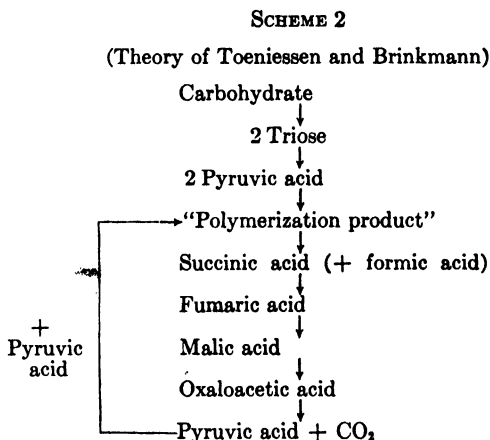
**The Theory of Thunberg and Knoop.**—The first attempts to formulate a scheme of carbohydrate oxidation are found in the papers of Thunberg (1920) (4) and of Knoop (1923) (120). The scheme which emerged from these papers is the following:



This scheme formulates an unbroken pathway for the oxidation of carbohydrate and offers an explanation for the formation of succinate from carbohydrate, pyruvate and acetate. It also accounts for the presence of certain enzymes in muscle. Its chief weakness was the complete lack of evidence supporting the assumption of a formation of succinate from acetate.

**The Theory of Toeniessen and Brinkmann.**—Toeniessen and Brinkmann (121) modified the scheme because they found that perfused muscle yielded some succinate (together with formate) when pyruvate was added to the perfusion liquid while acetate gave no succinate. This observation

led the authors to assume that the condensation occurs at the stage of pyruvate. They summarized their hypothesis in the following scheme:



The "polymerization product" of pyruvic acid was assumed to be 1,4-diketo-adipic acid:



The latter was thought to split into succinic and formic acids. Later investigators have confirmed the formation of succinate from pyruvate, but the formation of formate has not been reported again. The writer has never been able to detect a formation, or a decomposition, of significant quantities of formate in animal tissues and he considers it as probable that the formate in Toeniessen and Brinkmann's experiments was formed by bacteria. Toeniessen and Brinkmann admit that this was the case in some of their experiments on isolated muscle.

The scheme of Toeniessen and Brinkmann has recently been put to the test by Wille (122) who succeeded in synthesizing 1,4-diketo-adipic acid and added the substance to respiring muscle, kidney and liver. The rate of its decomposition in these tissues was found to be very low, about 10% of the rate of pyruvate decomposition and Wille comes to the conclusion that 1,4-diketo-adipic acid is unlikely to be an intermediate in the major pathway of carbohydrate breakdown.

In the light of more recently acquired knowledge, especially on transamination it is doubtful whether the succinate formed on addition of pyruvate (121, 28, 123) is actually synthesized from pyruvate. It may have been derived, via transamination from aspartic and glutamic acids.

**The Theory of Szent-Györgyi.**—Although the theory of Szent-Györgyi (70, 80) makes no attempt to depict the intermediate stages of carbohydrate oxidation, it may be discussed at this stage because of its close connection with oxidative processes in animal tissues. Szent-Györgyi offered an entirely new explanation for the presence in muscle of powerful enzymes catalyzing the oxidation of the four-carbon dicarboxylic acids. The new experimental findings on which Szent-Györgyi's conception was based, were the following:

1. Succinate (or its four-carbon oxidation products) was found to stimulate the respiration of muscle tissue catalytically.

2. Oxaloacetate is very rapidly reduced by muscle to a mixture of fumarate and malate (primarily to malate), even under aerobic conditions. The reaction  $\text{malate} \rightleftharpoons \text{oxaloacetate}$  is thus reversible. Although many dehydrogenations are reversible under some conditions, the rate of the hydrogenation is usually slow, or negligible, under the conditions prevailing in respiring muscle, *i. e.*, in the presence of molecular oxygen. They usually occur only anaerobically. The rapid reduction of oxaloacetate in the presence of oxygen therefore indicates a special significance of the reversible system  $\text{malate} \rightleftharpoons \text{oxaloacetate}$ .

On the basis of these observations Szent-Györgyi suggested that the main function of malate, fumarate and oxaloacetate in muscle "is not to serve as fuel, but to serve as a catalyst; as a catalytic hydrogen carrier between foodstuff and cytochrome" (124).

This assumption, which will be further discussed in relation to newer developments in Section II, 3, accounts for a number of observations, such as the catalytic effect of succinate and its breakdown products, and the occurrence of certain enzymes in muscle, but it is not, and is not intended to be, a complete theory of carbohydrate oxidation in muscle; it leaves unexplained the important observations discussed in the following section.

### 3. *The Citric Acid Cycle*

**Basic Experiments.**—The following four experimental observations led to the hypothesis of the "citric acid cycle" (Krebs and Johnson (42)):

1. The catalytic effect of citrate on the respiration of pigeon breast muscle which is of the same order of magnitude as that of succinate and its oxidation product found by Szent-Györgyi (70, 80) and by Stare and Baumann (6).

2. The rapid oxidation of citrate, isocitrate, *cis*-aconitate and  $\alpha$ -ketoglutarate in pigeon breast muscle ((Thunberg (4), Batelli and Stern (5), Krebs and Johnson (42)).

3. The synthesis of citrate from added oxaloacetate in muscle tissue (Krebs and Johnson, (42)). The yields of citrate, it is true, are comparatively low (amounting to no more than a few per cent of the added oxaloacetate) and, moreover, only if high concentrations of oxaloacetate (0.1 *M*) are employed, does citrate accumulate; but it was thought that this does not preclude an important role of the synthesis of citrate in muscle metabolism. Low yields may be due to the fact that the methods of separating the synthesis from the breakdown of citrate are imperfect. The rate of the accumulation of an intermediate is never more than a minimum rate of the actual formation of the intermediate. In fact it was found later (125) that  $\alpha$ -ketoglutarate—a breakdown product of citrate—arises together with citrate in very considerable quantities reaching 25% of the added oxaloacetate (81). The sum of citrate and  $\alpha$ -ketoglutarate formed on addition of oxaloacetate is thus very considerable. It ap-

TABLE VI

FORMATION OF SUCCINATE FROM OXALOACETATE AND FUMARATE IN THE PRESENCE OF MALONATE (126).

(The data refer to 267 mg. fresh pigeon breast muscle suspended in phosphate saline containing 0.025 *M* malonate and 0.02 *M* pyruvate; 40° C.; 70-min. incubation.)

Further substrates added (final concentration)	0.005 <i>M</i> fumarate	0.005 <i>M</i> oxaloacetate	0.005 <i>M</i> fumarate	0.005 <i>M</i> oxaloacetate
Gas	O <sub>2</sub>	O <sub>2</sub>	N <sub>2</sub>	N <sub>2</sub>
$\mu$ l. O <sub>2</sub> absorbed	884	595	0	0
$\mu$ l. succinate formed	306	254	43	39

proaches the maximum yield expected under the experimental conditions which were anaerobic; under these conditions part of the oxaloacetate acts as hydrogen acceptor and is reduced to malate.

4. The *oxidative* formation of succinate from fumarate or oxaloacetate. The *reductive* formation of succinate from fumarate and oxaloacetate can be inhibited by malonate as was shown by Szent-Györgyi and Gözsy (70), Krebs and Johnson (42) and by Krebs (126). Yet succinate can be formed from fumarate and oxaloacetate in the presence of malonate, provided that oxygen is available (see Table VI). These experiments show unequivocally that fumarate and oxaloacetate can be converted into succinate, although their anaerobic reduction has been essentially blocked by malonate. It follows that there are two pathways leading from the above two acids to succinate: direct reduction and an oxidative reaction so far not defined in detail. Barron and Stare (127), without giving reasons,

have stated that "this contention is without adequate proof;" but in the view of the writer the data given in Table VI and earlier data published by Szent-Györgyi (70, page 57) and Krebs and Johnson (42, page 104) have established the dual formation of succinate beyond doubt. Recent work on isotopes has confirmed the correctness of this view for pigeon liver (Wood, *et al.* (101)).

Among these four observations the last is the most important one. It shows that the oxidation of the four-carbon dicarboxylic acids leads to their re-formation; in other words, that there is a cycle of oxidations in which the dicarboxylic acids arise periodically. This is one of the basic facts which the theory of oxidations in muscle must take into account. It supplies at the same time a clear-cut hint regarding the nature of the intermediary oxidative processes in muscle.

The other three observations were taken by Krebs and Johnson to indicate the intermediary stages in the oxidative formation of succinate from oxaloacetate. Several authors have pointed out that catalytic effects of citrate could be explained on the basis of Szent-Györgyi's theory: the catalytic effect might be due to fumarate formed from citrate by oxidation. It is a serious flaw of such a hypothesis that it offers no explanation for the fact that citrate and its immediate breakdown products are readily metabolized in muscle, and it cannot be regarded as satisfactory if one takes the view, upheld by the writer, that this fact indicates that the substances in question are likely to be normal intermediates in muscle metabolism. This was an important consideration when the citric acid cycle was first proposed.

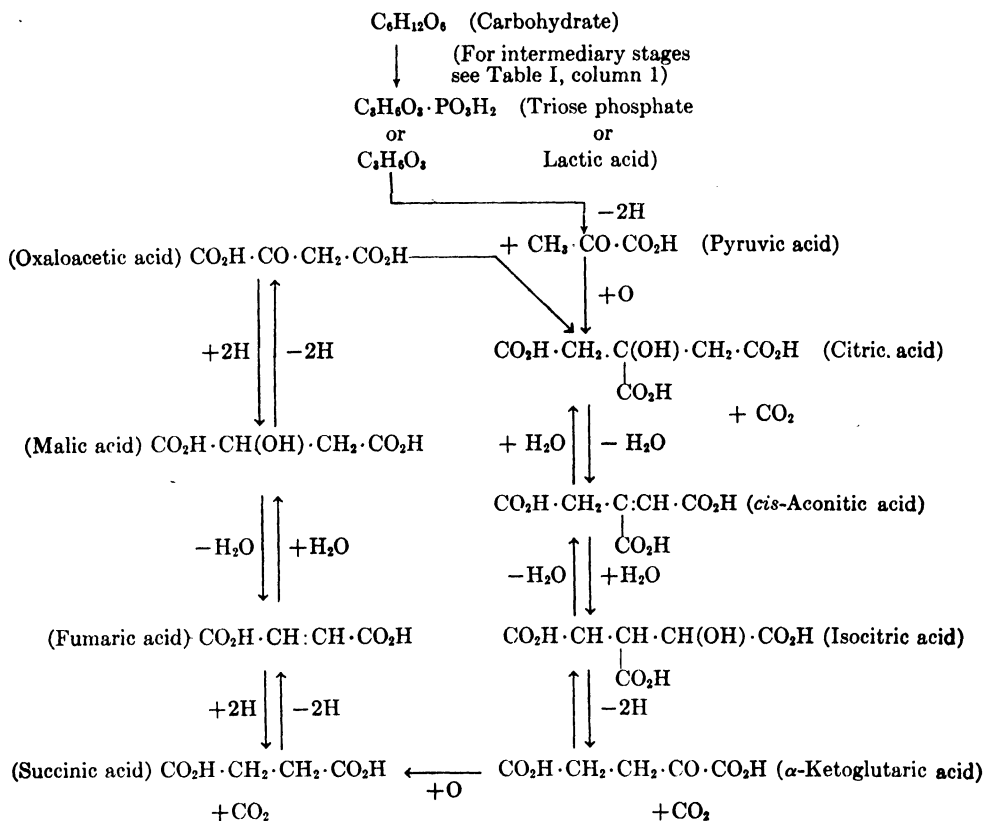
**The Earlier Scheme.**—The theory is contained in the reaction scheme on the following page.

According to the scheme carbohydrate is first split (anaerobically) to a three-carbon compound, either a triose phosphate or lactic acid. The oxidation of the three-carbon compound then yields pyruvate. The latter condenses with oxaloacetate to form citrate. Citrate in turn is oxidized eventually to yield oxaloacetate which becomes available to condense again with pyruvate and to repeat the "cycle."

The scheme offers a detailed, though not yet complete description of the intermediary stages of carbohydrate oxidation in muscle. It defines the major stages through which the carbon atoms of the carbohydrate molecules pass; it defines the steps where the actual oxidation occurs and the reactions by which carbon dioxide is released. The scheme explains the oxidative formation of succinate from fumarate and oxaloacetate. It "accounts," in fact, for the majority of the reactions listed in Table V

by allotting them a place in the process of carbohydrate oxidation. It also offers an explanation for the catalytic effects of citrate, succinate,

SCHEME 3. CITRIC ACID CYCLE



fumarate or oxaloacetate although the functions of the latter two substances, as will be seen later, are not yet fully described by the scheme.

**Discussion of Details.**—*Formation of Pyruvate from Carbohydrate.*—The theory suggests that, prior to the oxidation, carbohydrate is converted into three-carbon units and that pyruvate is the first oxidation product. Pyruvate may be formed from carbohydrate in different ways: either by glycolysis according to the Embden-Meyerhof-Parnas scheme

where it is an intermediate, or by re-oxidization of the lactate, formed by complete glycolysis, or by direct oxidation of hexoses, as discussed in Section II, 7.

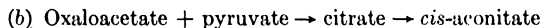
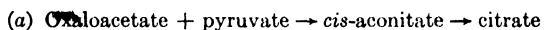
The last mode of pyruvate formation is probably the exception. It seems that, in general, hexoses are not oxidized as such, but only after anaerobic fission. This view is supported by two independent observations. First, inhibitors which prevent anaerobic glycolysis—iodoacetate or fluoride—usually inhibit also the oxidation of carbohydrate, although they do not inhibit the oxidation of lactate or pyruvate (Krebs (128); Meyerhof and Boyland (129); Ashford and Holmes (130); Colowick, Welch and Cori (131)). This may be taken to indicate that the anaerobic glycolysis precedes the oxidation. Second, no six-carbon oxidation products of carbohydrate have ever been observed in muscle. It is true, glucose can be oxidized to gluconic acid in liver (Harrison, 132) and glucose-6-phosphate can be oxidized to 6-phospho-hexonic acid in laked red blood cells to which methylene blue has been added (Warburg, Christian and Griese (133)). But these reactions have not been found to occur in muscle. There is one observation, however, which apparently argues against the view that glycolysis precedes oxidation: in brain cortex fructose is readily oxidized, although it cannot be converted into lactic acid (Loebel (134)). It remains to be examined whether in this tissue fructose is oxidized as such (see Section II, 7) or whether the inability to form lactic acid is merely due to an incomplete glycolysis ending perhaps at the stage of triose phosphate. But whatever the mechanism, experiments of Lipmann (87) show that the oxidation of fructose in brain also yields pyruvate.

*Condensation of Pyruvate and Oxaloacetate.*—When the theory was first formulated (42) it was left open which carbohydrate derivative condenses with oxaloacetate to form citrate. While the test tube experiments of Knoop and Martius (82) and of Claisen and Hori (83) favored the view that it might be pyruvate, it also appeared possible that another intermediate of glycolysis might react. Later it was found (8) that in minced muscle pyruvate is oxidized much more rapidly than, and in preference to, other substrates derived from carbohydrate. Moreover, when undergoing oxidation pyruvate reacts in accordance with the theory (Section I, 3). The facts are thus in agreement with the view that either pyruvate or a compound readily arising from pyruvate is the substance which condenses with oxaloacetate, while they are difficult to reconcile with the assumption that another carbohydrate derivative reacts with oxaloacetate. The assumption that phosphopyruvate is the reactive carbohydrate derivative is



so far unsupported by evidence; as far as is known pyruvate is not directly phosphorylated in suspensions of *minced* muscle.

*Order in Which Citrate, Cis-Aconitate and Isocitrate Arise.*—Citrate, *cis*-aconitate and isocitrate have so far always been found together in muscle. When citrate is synthesized from oxaloacetate and pyruvate the other tricarboxylic acids also arise, and it has therefore not been possible to decide in which order they are formed. It was made clear that the order chosen in the first schemes (42, 135) was somewhat arbitrary; it was uncertain, for example, whether *cis*-aconitate is a stage in the synthesis (scheme *a*) or in the decomposition of citrate (scheme *b*):



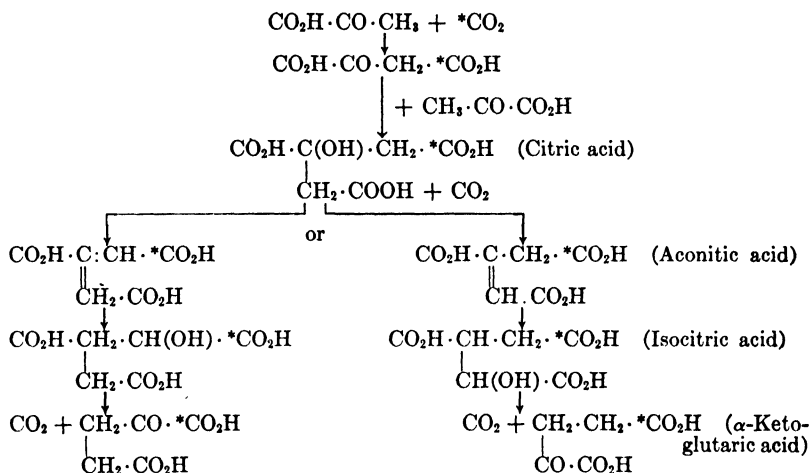
*In vitro* experiments offered no solution of the problem; for under some conditions—in alkaline medium—the condensation leads primarily to citrate (82), while under others—when potassium acetate is used as condensing agent—a derivative of aconitic acid is found (83). The primary products probably depend on whether the keto- or enol-form of oxaloacetate reacts, the former yielding the hydroxy acid, the latter the unsaturated compounds. Since neutral solutions of oxaloacetate contain both keto- and enol-forms (Meyer (136)) it is difficult to predict the course of the reaction.

The arbitrary formulation (b) was preferred (135) because it was thought for reasons already stated, that a place for the reaction,  $\text{citrate} \rightleftharpoons \text{cis-aconitate}$  should be found in the normal metabolism of muscle tissue (although this principle, of course, does not imply that the reaction is part of carbohydrate oxidation). Scheme (a) would offer no explanation for the ability of muscle to perform the above reaction. However, recent tracer experiments of Wood, Werkman, Hemingway and Nier (100) and of Evans and Slotin (102) indicate that scheme (b) is incorrect, at least in the case of pigeon liver, and that the scheme 3 must be modified. Pigeon liver, as already discussed in detail, synthesizes  $\alpha$ -ketoglutarate, citrate, fumarate and malate from pyruvate and carbon dioxide. For reasons given in Section I, 3, it can be taken that the synthesis of oxaloacetate is the first stage when pyruvate is converted into the di- and tricarboxylic acids. The further stages would be those described in the citric acid cycle. If this is correct the carbon dioxide fixed in the synthesis of oxaloacetate should be found in both carboxyl groups of the synthesized  $\alpha$ -ketoglutarate, as shown in the following scheme:

## SCHEME 4

SCHEME SHOWING THE FATE OF ASSIMILATED CARBON DIOXIDE BASED ON THE CITRIC ACID CYCLE

(The carbon atom introduced in the form of carbon dioxide is marked with an asterisk.)



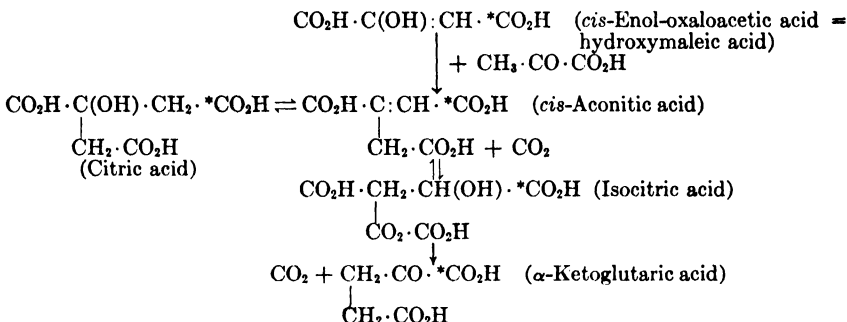
Owing to the symmetrical configuration of citric acid, as will be seen from scheme 4, the assimilated carbon is expected to appear in either of two carboxyl groups of aconitic, isocitric and  $\alpha$ -ketoglutaric acids. The prediction was tested with the isotopes  $\text{C}_{13}$  and  $\text{C}_{11}$  in the case of  $\alpha$ -ketoglutaric acid. The experiments confirmed only part of the prediction: the isotopes added in the form of bicarbonate were found to be present in  $\alpha$ -ketoglutaric acid and thus confirmed the participation of carbon dioxide in the synthesis of  $\alpha$ -ketoglutaric acid (see Section I, 3); but the distribution of the isotopic carbon was contrary to expectation. Only the carboxylic group adjacent to the ketonic group was found to contain the isotopes. This proved that the above scheme cannot be correct (for pigeon liver).

Wood, Werkman, Hemingway and Nier (100) have pointed out that a minor modification of the theory, already contemplated from the start (135) as one of the several possibilities, is in accordance with the facts. The main feature of the modification is the assumption that the condensation of (enol)-oxaloacetate and pyruvate yields primarily *cis*-aconitate which is directly converted into isocitrate while the formation of citrate is due to a side reaction shown on next page.

Provided that the rate of the side reaction between citrate and *cis*-aconitate is negligible compared with the rates of the other reactions, it is

## SCHEME 5

MODIFIED SCHEME SHOWING THE FORMATION OF  $\alpha$ -KETOGLUTARATE FROM OXALO-  
ACETATE AND PYRUVATE



expected from scheme 5 that the fixed carbon appears predominantly in the carboxyl group of  $\alpha$ -ketoglutaric acid adjacent to the carboxyl group, as is actually the case. Evans and Slotin (102) consider the modified scheme as "improbable in view of the demonstrated equilibrium between citrate, isocitrate and *cis*-aconitate in most tissues." The published data do, however, by no means indicate that the equilibrium between the three tricarboxylic acids was actually established under the conditions of the tracer experiments. Whether the equilibrium is established depends on the rates of the following five reactions:

- (A) *cis*-Aconitate  $\rightarrow$  isocitrate
- (B) Isocitrate  $\rightarrow$  *cis*-aconitate
- (C) *cis*-Aconitate  $\rightarrow$  citrate
- (D) Citrate  $\rightarrow$  *cis*-aconitate
- (E) Isocitrate  $\rightarrow$   $\alpha$ -ketoglutarate

If the rates of (A) and (E) are rapid compared with those of (B), (C) and (D) the quantities of citrate formed will be small, and the conversion of citrate into  $\alpha$ -ketoglutarate will be insignificant. Recent experiments of the writer (137) show that (A) and (E) are, in fact, much more rapid than are the other reactions. Martius (46) has already demonstrated that (A) is more rapid than (C). The writer finds (137) that at low concentrations (0.005 *M*) of *cis*-aconitate the isocitrate formation in the presence of aconitase is about nine times as rapid as the citrate formation. At higher concentrations the difference is not so great. As the physiological concentrations are even lower than 0.005 *M*, there can be no doubt that *cis*-aconitate formed as an intermediate in the tissue would yield predominantly isocitrate. Furthermore, added isocitrate yields much more

$\alpha$ -ketoglutarate and succinate in liver suspensions than added citrate, provided the concentrations are low and the experimental period is short (Table VII). This shows that reaction (E) must be rapid compared with (B), (C) and (D).

TABLE VII

## METABOLISM OF CITRATE AND ISOCITRATE IN PIGEON LIVER

(The data refer to 4 ml. liver suspension, containing 0.4 gm. liver; period of incubation: 5 min.; 40° C.; 0.025 M malonate.)

Substrates added	$\alpha$ -Ketoglutarate + succinate found	Citrate found
None	$0.45 \times 10^{-2}$ millimole	0 millimole
$4 \times 10^{-2}$ millimole isocitrate*	$1.35 \times 10^{-2}$ millimole	$0.19 \times 10^{-2}$ millimole
$2 \times 10^{-2}$ millimole citrate	$0.69 \times 10^{-2}$ millimole	$1.38 \times 10^{-2}$ millimole

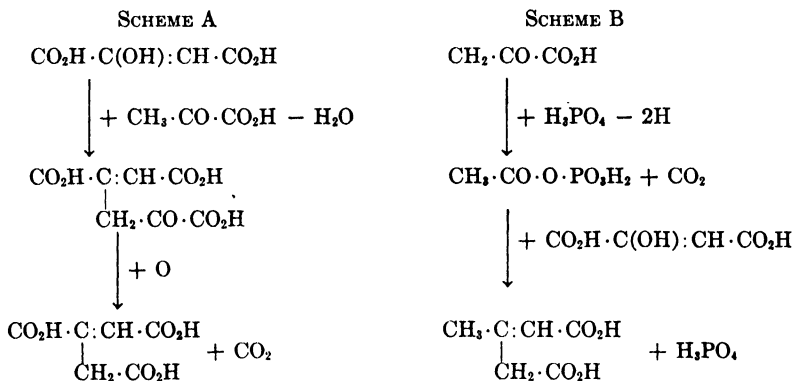
\* Isocitrate was a synthetic preparation of which 50% only is physiologically active.

These experiments indicate that owing to the relative slowness of the reactions *cis*-aconitate  $\rightleftharpoons$  citrate the equilibrium between the three tricarboxylic acids was not likely to be established in the tracer experiments on pigeon liver. Scheme 5 is thus not contradictory to the observed distribution of the fixed carbon in  $\alpha$ -ketoglutarate. It is true that a certain amount of fixed carbon must be present in the second carboxyl group if the theory is correct. It is not yet possible, however, to predict the quantities concerned. Under the conditions of the tracer experiments the rate of (A) is at least nine (probably many more) times as great as that of (C) and possibly 100 times greater than that of (D). If this is the case the expected abundance of fixed carbon in the second carboxyl group would be so small that its measurement would be beyond the accuracy of the experiments so far reported. Further work on the kinetics of the reactions (A) – (E) and on the position of the fixed carbon is desirable. Meanwhile scheme 5 may be provisionally accepted; it is the only available hypothesis which satisfactorily explains the facts.

The tracer experiments under discussion refer to pigeon liver. As it is highly probable that the mechanism of  $\alpha$ -ketoglutarate formation from oxaloacetate and pyruvate is the same in liver and in muscle, it is necessary to modify the citric acid cycle in accordance with scheme 5 by rearranging the order in which the tricarboxylic acids arise (see scheme 6).

*Mechanism of the Synthesis of the Tricarboxylic Acids.*—The formation of the tricarboxylic acids from oxaloacetate and pyruvate involves an oxidation which is equivalent to the removal of two hydrogen atoms. It cannot be definitely stated at what stage the oxidation occurs. It may precede, or it may follow, the condensation. In the former case the condensation would take place at the oxidation level of pyruvate and the condensation product (oxalo-citraconic acid\*) would subsequently be oxidized. In the second case pyruvate would be oxidized to the oxidation level of acetate before the condensation occurs. It is certain that acetate itself cannot be an intermediate, as its reactivity, if any, in muscle is far too low, but it has not been ruled out that acetyl phosphate ( $\text{CH}_3\cdot\text{CO}\cdot\text{O}\cdot\text{PO}_3\text{H}_2$ ) which according to Lipmann (138) is probably an oxidation product of pyruvate in *Bact. delbrueckii* can condense with oxaloacetate to form tricarboxylic acids.

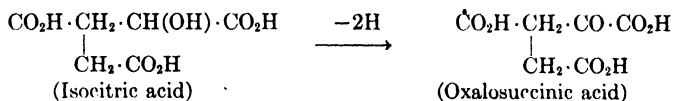
These two possible mechanisms are set forth in the following schemes:



At the present stage of knowledge both schemes appear possible. In favor of scheme A it can be pointed out that the reactions which it postulates have been shown to occur at least in the test tube while neither the formation of acetyl phosphate in muscle nor the ability of this substance to condense with oxaloacetate has so far been demonstrated.

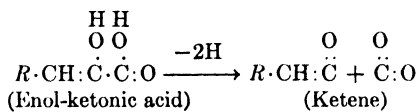
\* Wood, *et al.* (101) assumed the primary intermediate to be oxalomesaconic acid (or "pyruvofumaric acid"). This is the transisomeride of oxalocitraconic acid. If the resulting aconitic acid possesses the *cis*-configuration it is almost certain that the immediate precursors have the same configuration. This also applies to the enol-oxaloacetic acid in scheme 5 and in the following schemes (A) and (B) which is expected to be hydroxymaleic acid.

*Oxalosuccinic Acid as an Intermediate.*—The immediate oxidation product of isocitric acid is in all probability oxalosuccinic acid ( $\alpha$ -keto- $\beta$ -carboxylglutaric acid):

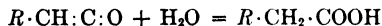


As a  $\beta$ -ketonic acid, oxalosuccinic acid is expected to decompose readily into  $\alpha$ -ketoglutaric acid and carbon dioxide. It has so far not been possible to test the metabolic behavior of the acid as its preparation has not yet been achieved. The acid is very unstable. When its triethyl ester is saponified, the resulting acid undergoes rapid ketone or acid decomposition.

*Mechanism of Decarboxylation of  $\alpha$ -Ketoglutarate.*—Two different mechanisms of decarboxylation have been discussed. Green, Westerfield, Vennesland and Knox (139) suggest that the decarboxylation precedes the oxidation and that succinic semialdehyde is an intermediary. They have prepared an enzyme preparation from pig heart and other animal tissues which, in fact, converts  $\alpha$ -ketoglutarate into succinic semi-aldehyde. The same enzyme converts pyruvate into acetyl-methyl-carbinol and  $\text{CO}_2$ . But as acetyl-methyl-carbinol is in all probability not a normal intermediate in animal tissues, it seems possible that the enzyme preparation of Green, *et al.*, is an artifact. Weil-Malherbe (140) suggests that the primary step is a dehydrogenation of the enol-form of  $\alpha$ -ketoglutaric acid and that a ketene is the intermediary:



The ketene would at once react with water to form succinic acid:



This scheme accounts for the fact that in most animal tissues—with the exception of liver—aldehydes are not readily metabolized (141, 142).

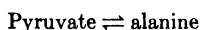
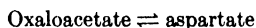
*Source of Oxaloacetate.*—According to scheme 3 the oxidation of carbohydrate depends on the presence of oxaloacetate. As the effect of oxaloacetate is catalytic the quantities required are expected to be small and to have no stoichiometric relation to the amount of carbohydrate oxidized.

Two sources of oxaloacetate are known. The food of all animals contains substances which yield oxaloacetate when broken down in the body:

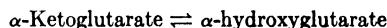
glutamic and aspartic acids, and other amino acids (see Section III, 2), citrate, succinate, malate. A second source is the synthesis of oxaloacetate from pyruvate and carbon dioxide. So far this reaction has only been found in the liver of the pigeon but it is likely to occur also in other tissues and organisms, though it seems to be absent from the suspensions of minced muscle commonly used in the study of oxidations.

*Side Reactions.*—If citrate is not an intermediate, but is formed by a "side reaction" the problem of the physiological significance of such a side reaction arises. At present little can be said about this. It is true that citrate, like many intermediary metabolites, has no doubt more than one part to play in the animal body. Its presence in relatively high concentrations in the testis (Thunberg, see (143)), in milk (see (147)), and especially in bone (Dickens (144)) and the relation of the urinary excretion of citrate to the menstrual cycle (145) indicate a variety of functions, but one would not like to assume that the supply of citrate for certain body liquids and tissues is the sole object of the citrate formation in muscle.

It is of interest that in addition to the reaction *cis*-aconitate  $\rightleftharpoons$  citrate there are in muscle three other rapid "side reactions"—the transamina-



and a further relatively slow reaction, *viz.*,



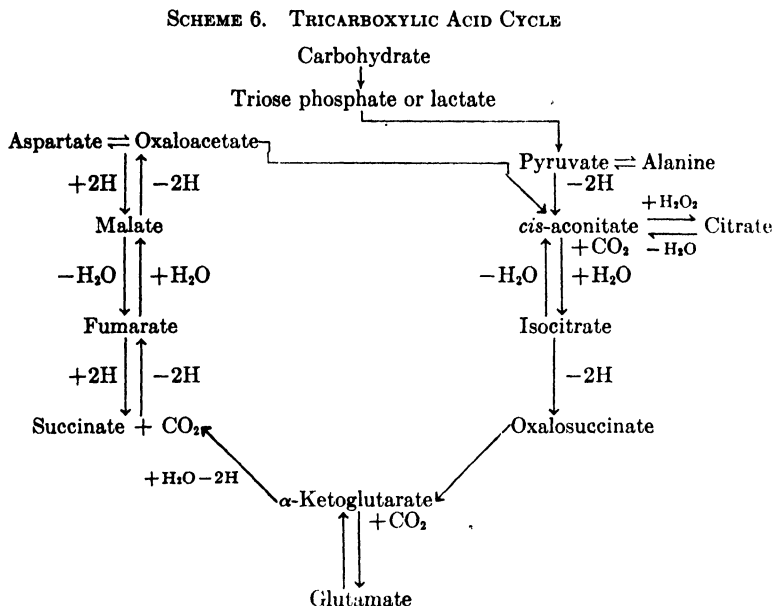
Maybe these reactions constitute a mechanism by which the tissue stores a reservoir of material from which it can readily draw oxaloacetate, when required as a catalyst. The presence of considerable amounts of glutamate and aspartate in muscle favors such a view, but it must be admitted that the reservoir of citrate is negligible in muscle.

Another "explanation" for the formation of citrate might be sought in the fact that *cis*-aconitate is an unstable substance which is rapidly converted into the metabolically inactive or even inhibitory (146) *trans*-form. The conversion into citrate would "protect" such *cis*-aconitate as cannot be immediately utilized in the cycle.

However, the writer wishes to make it clear that he regards these assumptions as merely tentative and that in his opinion nothing definite can be said about the significance of the various "side reactions."

4. *Modified Scheme (Tricarboxylic Acid Cycle)*

The modified and extended scheme of carbohydrate oxidation emerging from the preceding discussion is shown in the following scheme:



As far as the writer is aware this scheme is in accordance with all experimental observations made in experiments on pigeon breast muscle.

The criticisms which have been raised against the theory do not question the possible occurrence of the cycle, but the assumption that the cycle is the major mechanism of carbohydrate oxidation in pigeon breast muscle. These criticisms have been satisfactorily met. They are chiefly based on two observations: (1) the distribution of fixed carbon in  $\alpha$ -ketoglutarate (see Section II, 3) and (2) the relatively low rate of reaction of added citrate (Stare, Lipton and Goldinger (31)). The latter criticism was made against the original version of the cycle, but as added isocitrate and *cis*-aconitate show the same behavior (18) as added citrate—both oxygen uptake and substrate disappearance being smaller than postulated *under the conditions of the experiments of Stare, et al.*—the argument would also apply to the modified scheme. As already pointed out, the low rates of reaction are due to secondary inhibitions caused by the “de-ionization” of magnesium (see Section II, 2 and 3).



It is convenient to use a brief term for the theory describing the intermediate stages of the oxidation of carbohydrate in muscle. The essential feature of the theory is the periodic formation of a number of di- and tricarboxylic acids. As there is no term which would serve as a common denominator for *all* the various acids, one might name the cycle after one, or some, of its characteristic and specific acids. It was from such considerations that the term "citric acid cycle" was proposed in 1937. In the light of the recent work this name must now be regarded as inadequate. The writer proposes that it be replaced by the name "tricarboxylic acid cycle."

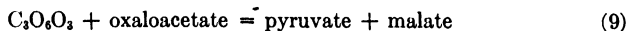
#### 5. *The Relations between the Szent-Györgyi Cycle and the Tricarboxylic Acid Cycle*

There is one major reaction in muscle which the scheme discussed so far does not explain: the reduction of oxaloacetate to malate. Szent-Györgyi, the discoverer of this reaction, explained it by the assumption that the

-2H  
system malate  $\rightleftharpoons$  oxaloacetate acts as a hydrogen carrier in respiration  
+2H

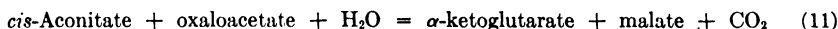
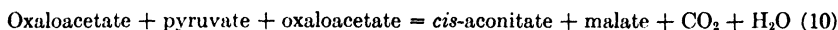
(see Section II, 2). The main piece of evidence supporting this hypothesis, in the view of the writer, is the fact that the postulated reactions can take place in muscle with great rapidity. Szent-Györgyi's second piece of evidence—the catalytic effect of the  $C_4$  dicarboxylic acids on respiration—carries less weight as the tricarboxylic acid cycle already accounts for such an effect.

A major aim in the further elaboration of Szent-Györgyi's hypothesis is the definition of the reactions by which hydrogen is transferred to oxaloacetate. Szent-Györgyi (80) and Parnas and Szankowski (147) defined one of these reactions; they showed that oxaloacetate is reduced when "carbohydrate" is oxidized to pyruvate. From the work of Negelein and Brömel (148) it appears that the actual hydrogen transfer in the oxidation of carbohydrate to pyruvate occurs when glyceraldehyde diphosphate is converted into diphosphoglyceric acid. It is probable, therefore, that it was this reaction in the experiments of Szent-Györgyi and of Parnas and Szankowski which was coupled with the reduction of oxaloacetate. Omitting the phosphorylations, the reaction of Szent-Györgyi can be written thus:



Two further reactions which donate hydrogen to oxaloacetate were found in the Sheffield laboratory. They are the stages in the tricarboxylic

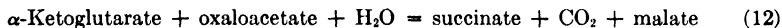
acid cycle leading from oxaloacetate and pyruvate to  $\alpha$ -ketoglutarate. These involve the transfer of two pairs of hydrogen atoms; the first arises during the synthesis of the tricarboxylic acids, the second during the oxidation of isocitrate to  $\alpha$ -ketoglutarate. Both these dehydrogenations occur anaerobically provided that an excess of oxaloacetate is available. The coupling of the dehydrogenation and hydrogenation in these two reactions may be expressed as follows (125):



The net effect of the three reactions (9), (10) and (11) is



Of the six pairs of hydrogen atoms released during the oxidation of one triose equivalent, three may pass through the stage of malate, while a fourth pair passes through the same stage during the further breakdown of  $\alpha$ -ketoglutarate. It is possible that oxaloacetate can also take part in the conversion of  $\alpha$ -ketoglutarate into succinate:



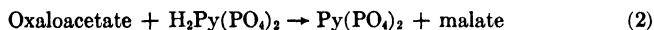
but this has neither been proved nor disproved. If reaction (12) occurs it would bring the number of hydrogen pairs possibly passing through the hydroxyl group of malate to five out of the total of six pairs.

It has been pointed out (149, 150, 151, 152) that there is some difficulty in understanding the physiological significance of the Szent-Györgyi system. Its participation in hydrogen transport would appear to be "pointless" (Ball (152)) if (as the available information suggests) diphosphopyridine nucleotide were the intermediate hydrogen carrier between molecular oxygen and malate as well as between oxaloacetate and the hydrogen of organic substrates. Ball (152) writes

"If we break the scheme into the separate reactions, this becomes evident. Assuming the substrate to be oxidized by means of diphosphopyridine nucleotide ( $\text{Py}(\text{PO}_4)_2$ ), we may write the first reaction as follows:



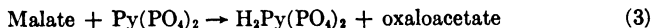
If the pyridine nucleotide is to act as a cyclic catalyst for this reaction, it must be oxidized. According to the Szent-Györgyi scheme, this reoxidation is brought about by oxaloacetate. The reaction may be written:



Now if the oxaloacetate in turn is to function as a catalyst, it must be regenerated.

\* For the experimental verification of this reaction see (81).

This requires that malate be oxidized. The oxidation of malate in the body, however, is known to proceed only through the diphosphopyridine nucleotide:



This equation is, however, the reverse of equation (2). Thus what is produced is reduced pyridine nucleotide, and we are right where we started when we wrote equation (1). The introduction of the malate-oxaloacetate system into this cycle merely leads us into a blind alley."

In the opinion of the writer it is, however, very unlikely that a reaction which can occur with very great rapidity is "pointless"; the problem therefore invites further investigation. It remains to be seen whether diphosphopyridine nucleotide is, in fact, the physiological hydrogen carrier in both the dehydrogenation of malate and the hydrogenation of oxaloacetate. Should this prove to be the case an explanation for the occurrence of the reversible reaction  $\text{malate} \rightleftharpoons \text{oxaloacetate}$  may be found in the assumption that the role of malate in hydrogen transport is analogous to that of creatine phosphate in phosphate transport, namely, to serve as a readily available reservoir of hydrogen.

It is certainly a most remarkable fact that muscle is equipped with a mechanism which enables the tissue to direct 8, and possibly 10, out of 12 hydrogen atoms of a triose equivalent to the hydroxy group of malate. The writer finds it difficult to believe that this elaborate mechanism serves no purpose. The mechanism is of particular interest in connection with the newer conception of energy utilization in muscle (see 153). The utilizable energy liberated from various anaerobic processes is assumed to accumulate in one compound, adenylypyrophosphate which appears to be the last link between the chemical energy of the substrates and the mechanical energy produced by the working muscle. It is probable that the energy derived from the various stages of the oxidations, like that derived from the anaerobic reactions, must also be canalized to one "center." Malic acid may be a link collecting the energy from various channels and directing it toward the "center," which is probably also adenylypyrophosphate.

In this connection Kalckar's discovery (154) of a formation of phosphopyruvate following the oxidation of malate in kidney extracts is of interest; the further elucidation of the mechanism of this reaction may be expected to throw light on the significance of the Szent-Györgyi cycle.

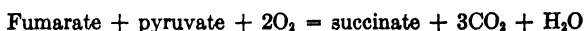
#### 6. Occurrence of the Tricarboxylic Acid Cycle in Various Materials

**Animal Tissues.**—The main experiments on which the theory of the tricarboxylic acid cycle is based refer to pigeon breast muscle and in the

first instance the theory applies to this tissue. Smyth who repeated the chief experiments with sheep heart (11) found no essential differences between the two tissues. Other materials have not yet been systematically examined, but a number of data have been recorded which seem to indicate that the cycle also occurs in other tissues. The relevant observations are the following:

1. Oxaloacetate yields citrate and  $\alpha$ -ketoglutarate in testis (sheep), kidney (guinea pig), brain (sheep) and liver (pigeon) (81).
2. The acids of the cycle are readily oxidized in kidney (78) and in liver (9). Other tissues (brain, testis) which under some conditions fail to show an increase in the oxygen uptake on addition of citrate, fumarate or malate, possess aconitase (47), fumarase (63) and malic dehydrogenase (69).
3. Certain brain preparations show an increase in the oxygen uptake on addition of citrate, fumarate and malate (155), and  $\alpha$ -ketoglutarate (156). They also show the catalytic effect of fumarate on the removal of pyruvate (157).

These observations make it clear that the various animal tissues which have so far been investigated possess essentially the enzymic equipment required for the tricarboxylic acid cycle and this strongly favors the view that the cycle occurs in the tissues in question. At the same time it must be pointed out that it has proved impossible (applying the same technique) to repeat some of the most convincing muscle experiments, for example, the demonstration of the reaction:



(see Section I, 3). But as already pointed out, negative experiments have a limited significance in this field of research. In the case under discussion this is especially true in the face of the positive results listed above. Negative experiments may be due to a partial destruction of enzyme systems as a result of the handling of the material; different tissues may behave differently in this respect. The history of the Embden-Meyerhof-Parnas theory of glycolysis teaches an impressive lesson on this point. A number of authors (158, 159, 160, 161, 162) found that they were unable to repeat with various other tissues the muscle and yeast experiments which established the theory for these materials. Especially no evidence showing the formation of phosphorylated intermediates was found. From such negative experiments it was concluded that a "nonphosphorylating glycolysis" occurred in many tissues. On further examination, however, Meyerhof and Perdigon (163) were able to repeat the decisive muscle ex-

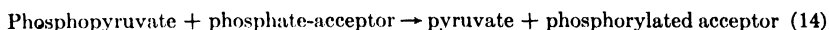
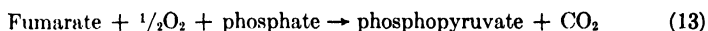
periments with the tissues in question and reviewing the subject Meyerhof (164) comes to the conclusion: "The so-called nonphosphorylating glycolysis is nonexistent except in imagination."

While thus there is evidence indicating the general occurrence of the cycle in tissues, other experiments (already mentioned in Section I, 3) show that in various tissues pyruvate undergoes reactions which are not included in scheme 6. The main reactions known so far are the formation of acetate, acetoacetate and  $\beta$ -hydroxybutyrate from pyruvate. Further experimental work is required before the relative significance of these reactions in various tissues can be assessed.

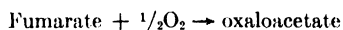
**Microorganisms.**—Pure cultures of bakers' yeast do not appreciably oxidize added citrate, isocitrate, *cis*-aconitate, succinate, fumarate or malate (68). The dehydrogenases activating these substrates have never been encountered in yeast. Fumarase (62) and aconitase (18) are also absent. The assumption of Lynen and Neciullah (165) that the above acids are intermediates in the oxidation of carbohydrate in yeast is therefore hardly tenable.

*Escherichia coli* and many other bacteria do not metabolize the three tricarboxylic acids. Organisms which attack citrate, *e. g.*, *Aerobacter aerogenes*, appear to employ a mechanism different from the tricarboxylic acid cycle (35, 43, 44, 45). There is therefore no support for the assumption that the cycle occurs in those bacteria whose intermediary metabolism has been studied in detail. On the other hand, many bacteria oxidize succinate, fumarate and malate and reduce anaerobically oxaloacetate to succinate and this suggests that the Szent-Györgyi system is operative in bacteria. Of special interest is the fact that catalytic effects of the four-carbon dicarboxylic acids have been found in bacteria where the tricarboxylic acid cycle or an analogous cycle does in all probability not occur, at least not under the conditions of the experiments. As was already pointed out the catalytic effects in animal tissues can be explained by the tricarboxylic acid cycle and therefore no "direct" evidence of the actual occurrence of the Szent-Györgyi cycle in animal tissues is available. The evidence only proves the *possible* occurrence of the cycle. In contrast the catalytic effect of fumarate, malate and oxaloacetate, under anaerobic conditions, on the fermentation of glycerol by propionic acid bacteria (73), or the dismutation of pyruvate in *Staphylococcus* (117) and *Escherichia coli* (27) can so far be explained only by the assumption that a cycle of the type visualized by Szent-Györgyi plays a role in these fermentations. In a number of bacteria (*e. g.*, *Escherichia coli*) the catalytic cycle apparently includes the reaction succinate  $\rightleftharpoons$  fumarate. The recent work on the

carboxylation of pyruvate in conjunction with Kalckar's (154) discovery of the formation of phosphopyruvate from fumarate suggests for certain cells a further extension of the cycle comprising the following reactions:



The net effect is:



and



These considerations indicate a possible coupling between the Szent-Györgyi cycle and phosphorylations.

**Molds.**—It is suggestive to assume that the formation of citric acid and of the four-carbon dicarboxylic acids in various molds from carbohydrate follows the same pathways as in pigeon liver. Tracer experiments show, in fact, that carbon dioxide is fixed in the synthesis of fumaric acid in *Rhizopus nigricans* and of citric acid in *Aspergillus niger* (166), and this supports the view that the mechanisms of fumarate and citrate formation are similar in pigeon liver and in molds. Little can be said about the mechanism of citrate decomposition in *Aspergillus*. Whether the pathway follows that in animal tissues (via  $\alpha$ -ketoglutarate), or that suggested for *Aerobacter* (via oxaloacetate and acetate (35, 43, 44, 45)) remains to be seen. Unlike bacteria and animal tissues molds can form oxalic acid in the course of the breakdown of citric acid.

**Plant Material.**—A number of the acids of the tricarboxylic acid cycle especially citric, succinic and malic acids, are encountered in many plants, frequently together with the acid amides of glutamic and aspartic acids. It has, moreover, been shown that the acids are interconvertible (167) in some plant tissues, that aconitase and fumarase occur and that in plants, as in muscle, citrate can be broken down to  $\alpha$ -ketoglutarate (39). Chibnall (168) points out that the interconversion may possibly take place through the tricarboxylic acid cycle and that the cycle may supply the carbon skeletons for glutamine and asparagine. The facts are in agreement with this view (see also Vickery and Pucher (169)) but the positive evidence is altogether scanty. Chibnall makes it clear that "the scheme must be regarded at present as nothing more than a convenient, if speculative, working hypothesis" (see also (170)).

### 7. Alternative Schemes of Carbohydrate Oxidation

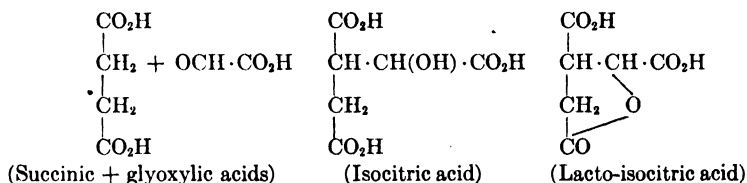
As was pointed out in the previous section there are various types of cells where the tricarboxylic acid cycle probably does not occur (such as yeasts and bacteria) or where this cycle does not seem to be the only pathway of carbohydrate oxidation (such as those animal tissues which form acetate from pyruvate). There can be little doubt, therefore, that further mechanisms of carbohydrate oxidation exist. The nature of these is obscure. The schemes which have so far been discussed are essentially speculative.

**Acetate as an Intermediate.**—It is reasonable to assume that one of the alternative paths of carbohydrate oxidation branches off at the stage of pyruvate leading to the formation of acetate. It is not impossible, however, that in some materials the formation of acetate might be an "artifact" due to the instability of acetyl phosphate (as methylglyoxal has proved to be an "artifact" due to the instability of glyceraldehyde phosphate).

So far all attempts to elucidate the metabolic breakdown of acetate in higher organisms, in yeast or in the common bacteria have been unsuccessful. Kleinzeller (105) using guinea pig kidney cortex—an animal tissue showing a high rate of acetate oxidation ( $Q_{\text{acetate}} = -8$ )—investigated the oxidation of the simple two-carbon compounds which might possibly be expected to be intermediate in the oxidation of acetate-glycolic acid, glyoxylic acid, oxalic acid, glycine but the low reactivity (if any) of these substances shows that they cannot be intermediates. This is also true for *Escherichia coli*. The inference is that the oxidation of acetate must involve a condensation with some other substance. This might be a phosphorylation, or a reaction of the type leading from oxaloacetate and pyruvate to the tricarboxylic acids. One condensation reaction of acetate is already known: the synthesis of acetoacetate, but as the decomposition of acetoacetate (in kidney cortex or in *Escherichia coli*) is much slower than the oxidation of acetate, this type of condensation is in all probability not a step in acetate oxidation.

While the negative results quoted so far exclude certain pathways, a few positive clues may be found in the following observations. Acetate oxidation in kidney slices is inhibited by malonate. Succinic dehydrogenase is therefore likely to play a part in the oxidation of acetate in kidney. Furthermore, the substances which kidney cortex can oxidize at a rate comparable to that of acetate are the acids of the tricarboxylic acid cycle, though knowledge on this point is incomplete, for not every possible intermediate has as yet been tested.

Basing a working hypothesis on these observations one might tentatively assume that the mechanism of acetate oxidation in kidney cortex may be somewhat similar to the tricarboxylic acid cycle involving, at least in part, the same intermediates. As already mentioned, acetyl phosphate might condense with oxaloacetate to form *cis*-aconitate. The difference between kidney (which oxidizes acetate) and muscle (which does not oxidize acetate) would then be due to differences in the ability to phosphorylate acetate. An alternative hypothesis is a modified Thunberg-Knoop scheme (scheme 1, page 221), proposed by Lipmann (153) who suggests that succinate may be formed by condensation of two molecules of acetyl phosphate. Test tube experiments point to a third possibility: succinate can condense with aldehydes, as was shown by Fittig (171), to form  $\gamma$ -lactones related to isocitric acid. If the aldehyde is glyoxylic acid the condensation is expected to yield lacto-isocitric acid:



One can thus picture a cycle in which isocitrate is oxidized to succinate, and re-formed by condensation with glyoxalate. Glyoxalate itself has proved to be metabolically inactive, or even inhibitory in the concentrations tested (172, 105). This makes it appear doubtful whether glyoxalate is an intermediary metabolite, but it remains possible that a related compound reacts in an analogous manner.

These considerations, it should be made clear, do not apply to yeast where so far no clues to the mechanism of acetate oxidation are available. Wieland and his collaborators (173) claim to have shown that yeasts can convert acetate into succinate and citrate. Even if this were correct, which is doubtful (174), it would not solve the problem of acetate oxidation. For both citrate and succinate are far too stable in yeast to be intermediary metabolites in a major oxidative process.

*Direct Oxidation of Hexoses.*—Dickens (175) and Lipmann (176) suggested a scheme of carbohydrate oxidation in which the first stages are the formation of hexose-6-phosphate and the oxidation of this ester first to 6-phosphohexonate, then to 2 keto-phosphohexonate and the decarboxylation of the latter to a pentose phosphate. A continuation of this process would finally yield pyruvate.



The following observations form the experimental basis of this scheme:

1. Hexose-6-phosphate is oxidized to 6-phosphohexonate by enzymes prepared from yeast (Warburg, Christian and Griese (133)).

2. Phosphohexonate reacts in the presence of yeast enzymes with 2.5 molecules of oxygen and forms three molecules of carbon dioxide (Warburg and Christian (133)).

3. Under suitable conditions the oxidation of phosphohexonate by yeast enzymes yields products giving Bial's test for pentoses and also analytically pure compounds corresponding to a phosphorylated four-carbon monocarboxylic acid (? phosphoerythronic acid) (Dickens (175)).

4. Yeast extracts oxidize *d*-ribose-5-phosphate vigorously while *d*-arabinose-5-phosphate and xylose-5-phosphate react slowly (Dickens (175)).

Dickens points out that according to his scheme *d*-arabinose-5-phosphate is expected to be the intermediate, and the fact that *d*-ribose-5-phosphate should prove to be much more readily oxidizable, introduces a new complication which so far has not been studied by experiment.

The scheme is obviously not an alternative to the tricarboxylic acid cycle, as it ends where the cycle starts: at the stage of pyruvate. The scheme outlines a pathway from carbohydrate to pyruvate alternative to glycolysis or fermentation. It may prove a valuable working hypothesis in the study of those special cases where hexoses are apparently oxidized without undergoing a primary glycolytic breakdown. Examples are the oxidation of fructose in brain (134), the oxidation of hexose-6-phosphate in lysed red blood corpuscles treated with methylene blue (133), bacteria which oxidize sugar without fermenting it (177) and possibly yeast poisoned with iodoacetate (178).

### III. Problems Related to the Oxidation of Carbohydrate

#### 1. Introduction

Knowledge acquired in the study of the intermediary stages of carbohydrate oxidation may be expected to shed fresh light on some of the general problems in the field of carbohydrate metabolism. Such problems are, for example, the nature of diabetes mellitus, the action of insulin, glucogenesis, antiketogenesis, the mechanisms of the Pasteur effect, the action of vitamin B<sub>1</sub>.

In a few instances it can be said that the expectations have been fulfilled and progress toward the solution of the problems has been made. In other cases the newer knowledge has contributed little or nothing toward

the solution of the outstanding problems—a reminder of the fact that many aspects of the intermediary carbohydrate metabolism are still obscure.

The following discussion is confined to those problems which in the view of the writer can be reviewed profitably at the present stage of knowledge and which have not been fully reviewed elsewhere.

## *2. Interrelations between Protein and Carbohydrate Metabolism*

**Glucogenesis from Carbohydrate.**—A number of amino acids are known to yield breakdown products in the animal body which also arise from, and can be resynthesized to, carbohydrate. Glutamic acid, aspartic acid and alanine, as already mentioned, form ketonic acids which are intermediates in the tricarboxylic acid cycle. Histidine (179), arginine (180, 181), citrulline, proline (180, 182-184), hydroxyproline (182, 183, 185) and probably lysine (184) all form glutamic acid in liver or kidney. In the case of histidine glutamic acid is formed hydrolytically under the influence of histidase, formyl glutamine and glutamine probably being intermediates (Edlbacher and Kraus (179)). The intermediary stages of the formation of glutaminic acid from arginine, citrulline, proline and hydroxyproline are not known in detail. The two former amino acids first form ornithine. The oxidation of ornithine and proline probably yields  $\alpha$ -keto- $\delta$ -amino-valeric acid (180) and then  $\alpha$ -ketoglutaric acid. As regards lysine the findings of Borsook and Dubnoff (184) strongly suggest a conversion into glutamic (or aspartic) acid, but some doubt about the occurrence of this reaction remains since lysine has not been found to be glucogenic. As glutamic and aspartic acids are glucogenic, amino acids which yield these acids should likewise be glucogenic. This is the case with arginine, proline, hydroxyproline and histidine (185, 186). The fact that the glucogenic action of histidine has been overlooked by the earlier workers (see Remmert and Butts (186)) makes a reinvestigation of the glucogenesis from lysine desirable. But even negative results would not necessarily invalidate the conclusion of Borsook and Dubnoff as a measurable glucogenesis can be expected only if the rate of glutamic acid formation from lysine reaches a certain critical level.

It becomes clear from this discussion that glutamic acid and  $\alpha$ -ketoglutaric acid are most important links through which the oxidative breakdown of protein is connected with that of carbohydrate. Altogether at least eight, and probably nine amino acids yield pyruvate on oxidation and the problem of glucogenesis from these amino acids is thus reduced to the problem of the "re-synthesis" of carbohydrate from pyruvate. The latter problem can be formulated in a comparatively simple manner. It amounts

to the question of how pyruvate can be converted into phosphopyruvate (Meyerhof (187)). Reactions leading from phosphopyruvate to carbohydrate are already known as the stages of glycolysis between carbohydrate and phosphopyruvate are all reversible (see Table I, column 1).

While many details are still obscure, it is already possible, as the preceding considerations show, to visualize, in broad outline, the intermediary stages of the conversion of part of the protein molecule into carbohydrate.

**Tricarboxylic Acid Cycle and Oxidation of Protein.**—The fact that eight or nine amino acids yield either  $\alpha$ -ketoglutarate, or oxaloacetate or pyruvate implies that the tricarboxylic acid cycle also plays a role in the oxidation of a considerable fraction of protein. In the case of casein, for instance, the eight amino acids concerned constitute more than 43% of the protein molecule. If lysine is added the figure reaches 48%. Protein can thus serve as a source of energy in muscle tissue. Three amino acids of the protein molecule—glutamic acid, aspartic acid, alanine—can be utilized directly. Part of the molecule, and most of the energy, of five or six other amino acids become available after conversion in liver and kidney into glutamic or  $\alpha$ -ketoglutaric acids. It seems that there is some design in the fact that the pathways of the metabolic decomposition of so many amino acids all join the same route: the conversion into the ketonic acids of the tricarboxylic acid cycle appears as a reaction which prepares amino acids for the utilization by muscle or brain, or other tissues.

### 3. *Interrelations between Fat and Carbohydrate Metabolism*

**Conversion of Fat into Carbohydrate.**—Although in diabetes mellitus or in depancreatized or phlorizine-treated animals, ingested fat does not appear as sugar in the urine, other more direct methods of experimentation clearly show the mammalian liver can form carbohydrate from fat (Gemmill and Holmes (188); Blixenkrone-Møller (189)).

Blixenkrone-Møller suggests that butyric acid arising from fatty acids undergoes  $\gamma$ -oxidation and is converted into succinic acid which is already known to be glucogenic. No other hypothesis has as yet been advanced. If the mechanism proposed by Blixenkrone-Møller is correct yet another metabolic pathway, that of the oxidation of fatty acids, joins the tricarboxylic acid cycle, and the principle discussed in the preceding section, according to which liver prepares substrate molecules in such a way as to make them suitable for the utilization by other organs, would also apply to fatty acids.

**Antiketogenesis.**—The earlier assumption (Dakin, Shaffer) that the antiketogenic effect of carbohydrate and other substances is due to a

chemical interaction between the ketone bodies and a breakdown product of carbohydrate has not been substantiated by the newer developments. No link between carbohydrate and ketone body breakdown has been found; in view of the fact that nothing is known about the mode of breakdown of acetoacetic acid, the failure to discover such a link does not, of course, exclude its existence.

A different conception of the phenomenon of antiketogenesis is now gradually emerging from various investigations. While the earlier hypothesis assumed that antiketogenic substances intervene in the *decomposition* of ketone bodies, it is now suggested that they prevent their *formation*, by way of a competitive reaction: substances are antiketogenic if they are oxidized, in liver, in preference to fat. Accumulation of ketone bodies in the body is assumed to be due to the fact that the capacity of the body for utilizing ketone bodies is limited and is smaller than the maximum capacity of the liver for producing ketone bodies. The latter capacity is, however, not fully employed as long as other oxidizable substances are available. It comes into play in starvation, or in diabetes, when carbohydrate cannot be utilized. The first to express this view, though in a crude, entirely speculative and not altogether acceptable form, was Macallum (190). The hypothesis was elaborated and provided with an experimental basis by Edson and the writer (191) in 1936. It was shown that substrate competition is a general phenomenon when several oxidizable substances are available (see Section I, 1). Further evidence supporting this explanation of antiketogenic effects has more recently been adduced by Mirsky (192) and by Stadie (193).

A more detailed analysis of the mechanism of substrate competition must await the results of further investigations of the enzyme systems concerned with the formation of acetoacetate. It is possible, however, from what is already known about the general structure of dehydrogenating enzyme systems, to picture the general pattern of the mechanism of competition. Competing systems presumably have one component, possibly a co-enzyme, in common. The total quantity of this compound limits the total oxidizing capacity of the systems, while the relative affinity of the common factor for the various enzyme systems determines the proportions in which various substrates are oxidized.

Edson (191) points out that apart from substrate competition there may be other mechanisms controlling ketogenesis; for instance, the powerful antiketogenic effects of glycerol in liver slices may require some special explanation.

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# THE CHEMISTRY AND BIOCHEMISTRY OF PANTOTHENIC ACID

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### I. Name

The name pantothenic acid (Gr. from everywhere) was given (1) because of the ubiquitous occurrence of the principle, before it was definitely known to be a vitamin. The retention of the name and the failure to use any letter-number designation for it marks an early departure from the traditional alphabetical vitamin nomenclature, and one which is almost certain to be followed in the future. It has been suggested that the name of the principle can, for certain purposes, be shortened conveniently to pantothen (2).

## II. Isolation

Because of its hydrophylic and polyfunctional nature, making precipitation and crystallization difficult, and because methods have not been devised for isolating *acids* of this type, the isolation of pantothenic acid from natural sources in entirely pure form has never been accomplished. Five years after the original paper (1), Williams and co-workers (3) announced (1938) the isolation of about 3 gm. of 40% pure calcium salt and small quantities of over 90% pure salt, from 250 kg. of sheep liver.

The process involved (a) autolyzing liver to obtain a clear filtrate, (b) removal of bases by adsorption on fuller's earth, (c) adsorption of the principle on charcoal and subsequent elution, (d) evaporation to dryness on kieselguhr in the presence of brucine and brucine oxalate, (e) extraction of the residue with dry chloroform to obtain brucine pantothenate mixed with other brucine salts, (f) a long and laborious procedure, using fractional distribution of the brucine salts between chloroform and water, (g) conversion to the calcium salt, (h) fractionation of the calcium salts with various solvents and solvent mixtures.

The final material was about 11,000 times as potent as a rice bran preparation which originally had been a starting material, and was retained as a "standard."

Three other groups of workers independently reported progress in the isolation of what ultimately proved to be pantothenic acid.

Koehn and Elvehjem (4) in concentrating the "antipellagra factor" for chicks (1937) obtained a preparation which contained 40% of the vitamin present in the original liver extract, and only 0.64% of the solids. This represented a 62-fold concentration starting with the liver extract. When doses of 0.7 mg. per day were fed to chicks, pellagra was prevented and the chicks gained 67 gm. more than those on the control diet, in 6 weeks. Though exact evaluation is impossible, it appears that the preparation may have contained 5%, and possibly more, of pantothenic acid. In 1938 Woolley, Waisman, Mickelsen and Elvehjem (5) reported regarding this same material "no increase in potency has been obtained." The same preparation contained nicotinic acid because it cured black-tongue in dogs (4).

Snell, Strong and Peterson (6) studying an "accessory factor for lactic acid bacteria" obtained in the same year (1937) a preparation which subsequent work shows to have been approximately 10% pantothenic acid. In 1938 (7) they indicated "a long series of fractionation procedures [applied to the 10% material] resulted in only about twofold increase in activity." Their "best preparation contained approximately 26% pantothenic acid."

The collaborators at the Lister Institute and the University of Manchester (1939) obtained pantothenic acid (designated by them "liver filtrate factor") in approximately 25% purity (8, 9).

Subsequent to the publication of Williams and collaborators (3) two other groups of investigators reported serious attempts to purify pantothenic acid.

Subbarow and Hitchings (10) by modifying Williams' procedure obtained "510 mg. of white varnish-like calcium salt (corresponding to Williams' fraction C)" [ca. 76% pure] from 160 kg. of liver. This preparation stimulated rat growth when administered at the rate of 8 mg. per rat per week.

Kuhn and Wieland (11) used as raw material 4 tons of tunny fish liver. By using (a) several precipitation procedures to remove inert material at various stages, (b) adsorption and elution using charcoal, (c) precipitation of the active principle with barium hydroxide in methanol, (d) chromatographic adsorption on alumina, they obtained material about 6% pure. They concluded that the active material was the same as that obtained from mammalian livers. Pure pantoic lactone was obtained from their concentrate by hydrolysis and, in addition,  $\beta$ -alanine, leucine and what appeared to be a homolog of pantoic lactone. Only the pantoyl- $\beta$ -alanine had physiological activity for *S. plantarum* which was used as a test organism.

### III. Recognition of Physiological Importance

The earliest recognition of a physiological effect which may be ascribed predominantly to pantothenic acid may be ascribed to Ide (12) [Wildiers (13)] who in 1901 found "bios" to be indispensable for the growth of yeasts. Under the conditions of the experiments reported, involving very low seeding and prolonged incubation time (Hansen I yeast), pantothenic acid is effective and is the only substance which appears to have chemical and physiological characteristics similar to the active principle studied. Biotin, for example, while an exceedingly potent yeast growth stimulant when tested for under appropriate conditions, is too stable for "bios," and does not show its effect when small seedings and long incubation periods are used. During long incubation periods, biotin is synthesized by the yeast rapidly enough to produce extensive growth (14). The recognition of pantothenic acid as a single effective nutrilitite did not come until 1931 (15) and 1933 (1).

The discovery of the effect of the principle now known as pantothenic acid on bacteria should be ascribed to Snell, Strong and Peterson (6, 7) who used lactic acid bacteria as test organisms in their concentration procedure, which resulted in highly potent material.

Possibly the first recognition of an effect on animal life due largely to pantothenic acid deficiency was that of R. R. Williams and R. E. Waterman (16) who discovered "vitamin B<sub>3</sub>," necessary for weight maintenance in pigeons. The effect observed was doubtless complicated by other factors as would necessarily be the case so early in the history of "vitamin B." A syndrome in chicks which is now known to be due, in a considerable degree, to pantothenic acid deficiency was discovered by Norris and Ringrose in 1930 (17). A condition resembling uncomplicated pantothenic acid deficiency more closely was produced by Kline, Keenan, Elvehjem and Hart (18) who introduced the heated diet into studies of chick nutrition. The diet of Norris, *et al.*, was deficient in a number of the members of the "B complex" which are required by young chicks, whereas

that of Kline, *et al.*, was almost lacking pantothenic acid and was deficient in riboflavin (19), as well as an unknown factor or factors (20, 21). The identity of the "chick antidermatitis factor" and pantothenic acid was strongly indicated by Woolley, Waisman and Elvehjem (22) who showed the former was a  $\beta$ -alanine derivative (like pantothenic acid), and by Jukes (23) who tested highly potent concentrates furnished by Williams and co-workers.

The "liver filtrate factor" required by rats (8) which was concentrated by the English workers, was later found to be replaceable by pantothenic acid (9) and it is clear that these workers were concerned with pantothenic acid as a physiological principle required by the rat. Subbarow and Hitchings (10) first reported pantothenic acid, as prepared by Williams and co-workers, to be a growth substance for rats. Morgan and co-workers (24) found graying of the fur of rats accompanied by adrenal and other lesions (25) to be due to "filtrate factor" deficiency and suggested the possibility that pantothenic acid might be involved. Negative indications, however, were obtained. György, Poling and Subbarow (26) obtained positive evidence with regard to the efficacy of pantothenic acid in curing the gray hair condition in rats.

#### IV. Chemical Structure and Synthesis

##### 1. Preliminary Findings

Long before pantothenic acid had been isolated or even concentrated, indirect experimental evidence was obtained indicating that its molecular weight was about 200; that it had in its structure no olefine double bond, aldehyde, ketone, sulfhydryl, basic nitrogen, aromatic or sugar group (1); that it was an acid with an ionization of about  $3.9 \times 10^{-5}$  (27) and possessed several hydroxyl groups (1) and a nitrogen atom with barely detectable basic properties (28). These extensive preliminary findings, which are unique in the history of the isolation of physiological principles, were made possible because a highly quantitative biological method for determining pantothenic acid could be used (1, 29, 30) and because of the extensive use of a relatively new tool, *i. e.*, fractional electrical transport (31).

##### 2. $\beta$ -Alanine as a Cleavage Product

The first definite step in the elucidation of the exact structure of pantothenic acid involved the discovery of  $\beta$ -alanine as a cleavage product (32, 33). Previous to this  $\beta$ -alanine had been found to be a yeast growth stimulant (34) when used in minute doses, and it later became clear that yeast used  $\beta$ -alanine as a "building stone" in the production of pantothenic acid.  $\beta$ -Alanine previously was known in the free condition and as a constituent of the peptides, carnosine and anserine, but its physiological significance was obscure. The combination of  $\beta$ -alanine in panto-

themic acid was found to be different from that in carnosine or anserine, because in pantothenic acid no amino group was present. In the two peptides the carboxyl group of the  $\beta$ -alanine is involved in the linkage while in pantothenic acid the amino group is involved.  $\beta$ -Alanine was first identified as a cleavage product of pantothenic acid because of its physiological effects upon yeast. Later it was isolated and identified as  $\alpha$ -naphthalene sulfo- $\beta$ -alanine (33).

### 3. Nitrogen-Free Portion of the Molecule

Evidence was obtained by Williams and co-workers that the other cleavage product of the pantothenic acid molecule was an  $\alpha$ -hydroxylactone in which the lactone was probably the  $\gamma$ -variety (35). Elementary analysis of the most potent pantothenic acid concentrates (36), however, had indicated that this lactone possessed five carbon atoms, instead of the six actually present. Later Williams and Major (37) announced that the lactone, as isolated in pure form in the Merck Laboratories from pantothenic acid concentrates, has the structure indicated by the name:  $\alpha$ -hydroxy- $\beta,\beta$ -dimethyl- $\gamma$ -butyrolactone. Simultaneously Woolley (38) reported obtaining a small amount of crystalline material thought to be the non-nitrogenous portion of pantothenic acid, but no analysis or structure was given.

The lactone derived from pantothenic acid for which the appropriate name pantoic lactone has been suggested (39) was not a new compound as it had long before been synthesized and obtained in racemic form by Glaser (40). The natural lactone, m. p. 91–92°,  $[\alpha]_D^{26} - 49.8^\circ$ , was isolated and its structure determined by degradation by Stiller, Keresztesy and Finkelstein (41) and was synthesized by Stiller, Harris, Finkelstein, Keresztesy and Folkers (42) following, with modifications, the work of Wessely (43) and Glaser (40) and Kohn and Neustädter (44).  $\alpha,\alpha$ -Dimethyl- $\beta$ -hydroxypropionaldehyde was prepared by aldol condensation of isobutyraldehyde and formaldehyde (43). This aldol was converted into its bisulfite compound and this into the cyanohydrin which was saponified to produce the lactone in good yield. The racemic lactone was resolved by fractional crystallization of the quinine salts, and the (+) lactone racemized in order to increase the yield of the desired (–) lactone, by heating the sodium salt in water solution.

Reichstein and Grüssner (45) and Carter and Ney (46) have developed modified procedures for preparing the lactone, involving the direct reaction of calcium chloride and sodium or potassium cyanide on the aldol in aqueous

solution, and Major and Finkelstein (47) have successfully used optically active quaternary ammonium bases, such as quinine methohydroxide, to resolve the lactone. Grüssner, Gätzi-Fichter and Reichstein (48) and Parke and Lawson (123) have assigned to the (–) lactone the *d* configuration.

#### 4. *Methods of Condensation*

Groundwork had already been laid for the synthesis of pantothenic acid as soon as the structure of the two constituent parts was known.

Williams and co-workers in June, 1938, before the presence of a  $\beta$ -alanine residue in the pantothenic acid molecule had been announced, had brought about a partial synthesis of pantothenic acid by condensing a  $\beta$ -alanine ester with the impure lactone obtained from a pantothenic acid concentrate; the ester so obtained was hydrolyzed to yield the physiologically active pantothenic acid (49). Similar partial syntheses were accomplished independently by Snell, Strong and Peterson (50), received February, 1939, and Woolley, Waisman and Elvehjem (51), received March, 1939, who were investigating an "accessory factor for lactic acid bacteria" and the "chick antidermatitis factor," respectively, from the standpoint of their relationship to pantothenic acid, which was now recognized as a  $\beta$ -alanine derivative of a dihydroxy acid (32).

There are two fundamental practical methods for the synthesis of pantothenic acid. One involves the reaction of pantoic lactone with a  $\beta$ -alanine ester. This was first used by Williams and co-workers (48, 49) using crude lactone from natural sources, and was developed on a practical scale using the synthetic lactone by Stiller and co-workers (42) and independently by Reichstein and Grüssner (45) and Kuhn and Wieland (52). In the early experiments of Snell, Strong and Peterson (50) and Woolley, Waisman and Elvehjem (22, 51) a modification of the ester-lactone condensation was used. Steps were taken to produce the acid chloride of the acetylated pantoic acid for use as a reactant, but since the acid chloride was not isolated it may well have been the acetylated lactone itself which condensed with the  $\beta$ -alanine ester. These experiments were carried out before the non-nitrogenous part of pantothenic acid was known to be a lactone which would itself react with  $\beta$ -alanine esters. Kuhn and Wieland (52) utilized the benzyl ester of  $\beta$ -alanine to react with pantoic lactone, and subjected the product to catalytic hydrogenation.

The use of  $\beta$ -alanine esters for the synthesis has two disadvantages. The simple alkyl esters, at least, are difficult to prepare or keep since they polymerize readily on standing (53). When an ester of  $\beta$ -alanine is used, the corresponding ester of pantothenic acid is produced, and a subsequent cleavage of the ester is necessary to obtain pantothenic acid, which is itself subject to hydrolytic cleavage under rather mild conditions.

The other general method of synthesis obviates both of the difficulties

mentioned above. It involves the reaction between the pantoic lactone and a salt of  $\beta$ -alanine to yield directly a salt of pantothenic acid. This was first carried out in alcoholic solution in Williams' laboratory, but a poor yield was obtained. Later when synthetic pantoic lactone became available, Williams, Mitchell, Weinstock and Snell (49) obtained a theoretical yield of sodium pantothenate by reacting dry pantoic lactone with sodium  $\beta$ -alanate. Independently Babcock and Jukes (54) carried out the reaction in concentrated water solution with good yields, and Woolley (55) proposed a synthesis along somewhat similar lines except that the unnecessary steps involving acetylation and the use of thionyl chloride to produce the acid chloride were included. Moore (56) has obtained pantothenic acid by condensation of pantoic lactone with free  $\beta$ -alanine.

### 5. Resolution

The resolution of pantothenic acid by use of its quinine salts was reported by Stiller, Harris, Finkelstein, Keresztesy and Folkers (42) simultaneously with their announcement of the total synthesis of pure pantothenic acid. As indicated above, the pantoic acid portion of the molecule bears the asymmetric carbon atom and the natural lactone is levorotatory. Free pantothenic acid has been obtained in the form of a pale yellow viscous oil with a rotatory power,  $[\alpha]_D^{26} +37.5^\circ$ . The calcium salt is likewise dextrorotatory,  $[\alpha]_D^{26} +24.3^\circ$ . Kuhn and Wieland (52, 57) also brought about the resolution of pantothenic acid by fractional crystallization of the quinine and cinchonidine salts, and Stiller and Wiley (58) used quinine methohydroxide for its resolution, as did Major and Finkelstein (47) for resolving pantoic lactone.

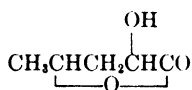
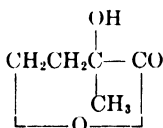
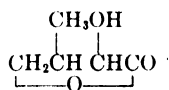
### 6. Specificity

When  $\beta$ -alanine was discovered to be a nutritive for yeasts (34) its action in this respect was found to be unique in that none of the common  $\alpha$ -amino acids, or such closely related  $\beta$ -amino acids such as  $\beta$ -amino butyric acid or isoserine,  $\text{CH}_2\text{NH}_2\text{CHOH}-\text{COOH}$ , had a physiological effect which was in any way comparable with that of  $\beta$ -alanine (59). Weinstock and co-workers (60) have recently condensed various amino acids including  $\alpha$ -alanine,  $\beta$ -aminobutyric acid, aspartic acid and lysine with pantoic lactone, and found the products to be biologically inactive. Kuhn and Wieland (11) found the leucine-pantoic lactone condensation product also to be inactive physiologically. These observations point to a definite biological



specificity so far as the  $\beta$ -alanine portion of the pantothenic acid molecule is concerned.

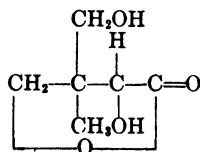
The non-nitrogenous portion of the pantothenic acid molecule can be altered in a number of ways without completely destroying the physiological potency. The optical antipode of natural pantothenic acid appears to have substantially no physiological activity either on microorganisms or on experimental animals (42); the configuration of the asymmetric carbon atom therefore cannot be inverted without destroying the physiological effectiveness. Among the lactones found in Williams' laboratories (35) to have some slight physiological activity when condensed with  $\beta$ -alanine, are the following:

 $\alpha$ -Hydroxy- $\gamma$ -valerolactone $\alpha$ -Hydroxy- $\alpha$ -methyl- $\gamma$ -butyrolactone $\alpha$ -Hydroxy- $\beta$ -methyl- $\gamma$ -butyrolactone

Subbarow and Rane (61) and Woolley and Hutchings (62) have reported that  $\alpha,\delta$ -dihydroxyvaleryl- $\beta$ -alanine is physiologically effective on certain strains of hemolytic streptococci but is required in much larger amounts than is pantothenic acid. Snell, Woolley and Strong (63) independently prepared and tested several of the  $\beta$ -alanine derivatives referred to above with similar findings. The activity for lactic acid bacteria (35) was in each case only a fraction of a per cent as great as that of pantothenic acid, but was nevertheless great enough to be readily detected. It may be noted that all of the compounds capable of yielding  $\beta$ -alanine derivatives with a trace of physiological activity, have an  $\alpha$ -hydroxyl group.

Reichstein and Grüssner (45) found  $\alpha,\gamma$ -dihydroxyvaleryl- $\beta$ -alanine and  $\alpha,\delta$ -dihydroxyvaleryl- $\beta$ -alanine to possess no more physiological activity for rats than free  $\beta$ -alanine.

Hydroxypantothenic acid, prepared by Mitchell and co-workers (64, 65) from hydroxypantoic lactone and sodium  $\beta$ -alanate, is definitely more active than any of the other known compounds closely related to panto-



Hydroxypantoic lactone

thenic acid. Its physiological activity varies from 1.5% to 25% of that of pantothenic acid depending upon the microorganism used and the conditions of testing. From this interesting fact it was deduced that probably hydroxypantothenic acid is not a naturally occurring substance, since preparations from natural sources do not vary in this manner when tested in comparison with synthetic pantothenic acid. Hydroxypantothenic acid was found to have physiological potency for rats but was an incomplete substitute for pantothenic acid (66).

In connection with the question of specificity, the finding of Kuhn and Wieland (11) of what was thought to be a homolog of pantoic lactone in the hydrolysis product of their pantothenic acid concentrate, is interesting, but the condensation product of this lactone and  $\beta$ -alanine did not possess physiological activity.

We must conclude that pantothenic acid, as it is found widespread in nature, is probably a single definite chemical substance.  $\beta$ -Alanine derivatives with closely related structures appear to have at most very slight physiological activity, or are incomplete substitutes for natural pantothenic acid.

It should be pointed out that substances which act as building stones for the production of pantothenic or which yield pantothenic acid on hydrolysis, may promote growth in a manner similar to pantothenic acid. Thus, for yeast,  $\beta$ -alanine serves as a precursor of pantothenic acid, but is in general much less effective even on an equal weight basis.  $\beta$ -Alanine is relatively effective in the presence of *low* concentrations of other amino acids (asparagine or aspartic acid), but is much less effective when the amino acid concentration in the medium is high (33). This is apparently due to a competitive action of other amino acids in preventing the absorption of  $\beta$ -alanine by yeast.  $\beta$ -Alanine also serves as a "growth substance" for the diphtheria bacillus (67). Pantothenic acid is effective at even lower concentrations and the evidence points clearly to the probability that  $\beta$ -alanine is a precursor of pantothenic acid.

Hoffer and Reichstein (68) in preliminary experiments found  $\beta$ -alanine to be physiologically effective for rats, but the importance of  $\beta$ -alanine in this connection has not been confirmed elsewhere (69, 70) or in Reichstein's laboratory (48).

Woolley (71) found that a strain of hemolytic streptococcus gave a growth response when pantoic acid without  $\beta$ -alanine was added to the culture medium. Again pantothenic acid itself was more effective and it was concluded that pantoic acid served as a precursor of pantothenic acid. Yeast is affected by relatively high concentrations of pantoic acid

in the presence of  $\beta$ -alanine, but too high concentrations were required to make it a useful test (72). Lactic acid bacteria, on the other hand, are, in general, not materially affected by moderate concentrations of either pantoic acid or  $\beta$ -alanine or hydrolyzed pantothenic acid which contains both (50).

Even animals are able to bring about some synthesis of pantothenic acid if the two fragments,  $\beta$ -alanine and pantoic lactone, are furnished in abundant quantities. Babcock and Jukes (54) reported that chicks responded definitely to such a mixture and calculated that roughly 0.06% coupling took place *in vivo*. Grüssner, Gätzi-Fichter and Reichstein (48) found that pantoic lactone (0.5 mg. per day) in the presence of  $\beta$ -alanine (0.5 mg. per day) promoted the growth of rats so that they gained 1.8 gm. per day as compared with 0.35 gm. per day for the controls. The question may be raised whether the synthesis of pantothenic acid in experiments such as these is being effected in the tissues of the animal or in the intestine by bacterial action.

The alkyl esters of pantothenic acid and their acetyl derivatives are ineffective as nutrilites for yeast or lactic acid bacteria. However, such derivatives are apparently hydrolyzed in the digestive tract of animals. Grüssner, *et al.* (48), found ethyl and methyl pantothenates to be effective in promoting the growth of rats and Unna and Mushett (73) found ethyl monoacetyl pantothenate and ethyl pantothenate to be as effective on rats as an equimolecular amount of calcium pantothenate. Ethyl monoacetyl pantothenate was also tested upon chicks and found to be fully effective. Woolley (74) found pantothenic acid diphosphate to be biologically inactive when tested on microorganisms.

Pantothenic acid as it occurs in natural foods is often in a combined form, and there are indications that it may be bound through an amide linkage to proteins (75). This combination certainly serves as an effective source of pantothenic acid, and digestive enzymes must cause its liberation. Bound pantothenic acid, like the esters and their acetyl derivatives, are, in general, ineffective for microorganisms and require preliminary liberation.

In connection with the problem of specificity, the "anti-vitamin" activity of pantoyl taurine discovered by Snell (76, 77) is interesting. This substance when introduced into the culture media for yeasts or lactic acid bacteria inhibits growth, but its effect is overcome by additional pantothenic acid. It appears that there is a competition between pantothenic acid and pantoyl taurine. When the ratio between the concentrations of pantoyl taurine and pantothenic acid is sufficiently high, pantothenic

acid is "blocked out" and growth ceases. This inhibition apparently is observed only in those organisms for which pantothenic acid is an essential nutriment. Its effects on animals is being studied.

## V. Origin-Distribution-Quantitative Determination

The fact that pantothenic acid appears to be everywhere in living matter, as the name suggests, has no bearing on the question of whether deficiencies exist. Recent studies (78, 79) indicate that pantothenic acid shares its universal presence in living matter with all the other B vitamins. Even though thiamin, for example, probably occurs universally, diets which are quantitatively deficient in thiamin are common.

Pantothenic acid has two types of origin both of which are probably important. Green plants under sterile conditions can, after the photosynthetic apparatus is functioning, produce pantothenic acid (80) and it must be assumed that this is an important source in the economy of nature. Various molds (1, 81), bacteria (79) and yeasts (82, 83) produce pantothenic acid when grown on a medium which is free from it. Some yeasts apparently require  $\beta$ -alanine as a building stone while others do not. Molds and bacteria may require a nutritional source of pantothenic acid or may be stimulated by it, but in any case it appears always to be present in the cells produced. The production of pantothenic acid in the soil by molds and bacteria may be important and its bacterial production in the rumen of herbivorous animals certainly is.

Rohrman, Burget and Williams (84) first made determination of the pantothenic acid content of various animal tissues, and noted the effect of autolysis. Jukes (86, 87), Jukes and Lepkovsky (85) and Bauernfeind (88) have given values for a number of foods and feed materials. Peterson and Elvehjem (82) have studied the content of yeasts; Waisman, Mickelsen and Elvehjem (89) meats and meat products. Strong and co-workers (90, 91) have assayed foods (and other materials) and found no great destruction in foods by cooking. Pearson (92) has determined the pantothenic acid content of blood of six species including human's. Waisman, Henderson, McIntire and Elvehjem (93) have found the pantothenic acid content of meats as determined microbiologically to be increased by enzymatic digestion. Wright and collaborators (94) have given assay values for various rat, mouse, beef and hog tissues (autolysates), and Williams and co-workers (95) have studied the content of tissues during embryonic development. These latter studies at the University of Texas have been extended, using enzymatic hydrolysis (96), to human tissues (97), diverse organisms (98), miscellaneous foods (99), bacteria (100), cell nuclei (101) and cancer tissues (102, 103). Miscellaneous additional information regarding the distribution of pantothenic acid will be found in articles referred to later in connection with assay methods. The very high content of "royal jelly" (104) is worthy of special notice.

The yeast growth method was used in the discovery (1) and isolation (3) of pantothenic acid but it was fully recognized, especially in the later stages of concentration, that the test was not entirely specific, when used on crude preparations. For testing potent concentrates it was highly specific and accurate (33) except for the  $\beta$ -alanine effect. When the method as applied to tissues was studied later it was abandoned in favor of a bacterial test, partly because of the possibility of  $\beta$ -alanine interference and partly because of the toxic effects of "old" extracts on yeast.

The methods involving the use of *Lactobacillus casei* are based upon the fundamental findings of Snell, Strong and Peterson (6, 7, 50) and have been developed by Pennington, Snell and Williams (105) and Strong, Feeney and Earle (91). The two methods give substantially the same results; in the latter case asparagin is added to the culture medium, but does not modify the assay values materially (91).

An ideal basal medium for these tests would be a completely synthetic one, but this is for the future, and it is necessary to introduce alkali-treated natural extracts (in which the pantothenic acid is destroyed). Various interfering substances may complicate the test (105, 106, 107, 108) but there are fairly satisfactory remedies for all the difficulties. The use of an alkali-treated extract of the material to be assayed, in the basal culture medium as suggested by Pennington, *et al.* (105), is an important expedient. Blood (or any material which is a very poor source of pantothenic acid) offers real difficulties, which, however, are not insurmountable (109).

Another bacterial method is based upon the observations of Pelczar and Porter (110) with regard to the pantothenic acid requirements of *Proteus morganii* and has been developed and applied by them to the assay of blood and urine (111, 112). Responses are obtained from 0.001  $\gamma$  pantothenic acid per 10 ml. culture. Their values for blood are a little more than one-fourth of those obtained by Stanbery, *et al.* (109), and Pearson (92) using the *L. casei* method. The cause of this discrepancy is not known but may have been due to differences in the preparation of the samples for analysis or to differences in the ability of the organisms to utilize the pantothenic acid as it occurs in the blood. The values obtained by this method for urine (average 3.81 mg. per day) agree substantially with those of Gordon (113) (3.52 mg. per day) and Pearson (114) (3.19 mg. per day average for 3 subjects) and Wright and Wright (115) (*ca.* 3.3 mg. per day), as determined by using *L. casei*.

The number of bacteria which are potentially usable for pantothenic acid testing is large. Among those used rather extensively beside the two mentioned are *Streptococcus lactis* (35) and *Streptobacterium plantarum* (11). The existence of alternative microbiological methods by which pantothenic acid may be determined is an advantage, because they can be used to check one another, and if one fails for certain materials others may be applicable. All microbiological methods have the advantage of being applicable to small amounts of material. The method of Pen-

nington, *et al.*, has been adapted to determine 0.0002  $\gamma$  to 0.001  $\gamma$  (total) of pantothenic acid (116).

The chick growth method has been used most extensively by Jukes (86, 87) and since it involves only a two-week growth period on young chicks (which are readily available) it is relatively simple compared with other animal assay methods. The results are in general in good agreement with those obtained by bacterial assay (*L. casei*), but when certain materials such as liver extract, rice bran extract and, particularly, yeast are tested, the values by the chick method are much higher than the values obtained by bacterial assay (116). György (117) has indicated that the chick assay method is more reliable, because of difficulties of extraction involved in the bacterial methods. The writer's interpretation is that the chick assay method in some cases gives much too high values, simply because of the presence of growth-promoting substances for chicks, other than pantothenic acid, in the materials tested. It is and has been well recognized that a heated diet such as is used in chick assays is low in at least one other principle needed by chicks, besides pantothenic acid (20, 118, 119, 120).

A chemical method for determining pantothenic acid has been rather thoroughly investigated by Thompson (121) at the writer's suggestion. The method was based upon the selective oxidation of pantoic lactone by lead tetraacetate and the hope that other lactones obtained from natural sources would not be of the type to be affected. The oxidation was found to be erratic and not very selective when applied on a micro scale to natural extracts and there seemed no hope of devising a method which would be useful at the present time.

During earlier study of pantothenic acid various "units" were employed for designating quantities. One "gram unit" (= 80  $\gamma$  Ca pantothenate) was employed by Williams and co-workers (3). A chick unit (= 14  $\gamma$  Ca pantothenate) was utilized by Jukes (86) and a streptobacterium unit (0.02  $\gamma$ ) was used by Kuhn and Wieland (11). Since pure salts are now available these units are of no value, and a quantity of pantothenic acid can best be expressed in terms of weight.

Calcium pantothenate was the first salt prepared; it is available in pure crystalline form and since it has been used extensively as a standard substance there seems to be no good reason for changing, though because of its greater ease of crystallization, Gätzi-Fichter, Reich and Reichstein (122), and Parke and Lawson (123) have suggested the use of the sodium salt. The equivalent weights of sodium and calcium are similar, so that weights expressed in terms of the two standards would be about the same.

## VI. Physiological Functioning

### 1. Fundamental Role or Roles

Among the first observations made with regard to the physiological functioning of pantothenic acid was that it stimulated the growth of alfalfa seedlings and caused an increase in carbohydrate production, without increasing the nitrogen assimilation (80). Later it was observed that glycogen storage in yeast was increased very definitely when pantothenic acid was supplied (124). These facts suggested that pantothenic acid probably plays some fundamental role in carbohydrate metabolism. As long as the effects are observed on living organisms, however, the question may always be asked: Is the effect direct or indirect; does pantothenic acid itself enter into the carbohydrate metabolism mechanism or does it affect the process by functioning in some other way necessary to the metabolic activity of the organism?

Pratt and Williams (125) reported that pantothenic acid had a slight definite effect on fermentation by dialyzed yeast maceration juice, but Teague and Williams (126) reported inability to confirm this observation. In the latter study the possibility of pantothenic acid entering into the fermentation process in the non-living system could not be ruled out, since no maceration juice capable of carrying on fermentation could be prepared, which did not contain combined pantothenic acid in appreciable amounts. It was concluded that pantothenic acid probably did not constitute a *dissociable* coenzyme, involved in any of the recognized steps. It may well be that there is some as yet unknown mechanism whereby pantothenic acid is *built into* an essential enzyme system, which is concerned with carbohydrate metabolism, and that failure to preserve this mechanism results in pantothenic acid becoming ineffective. In a living organism this hypothetical mechanism is always present and pantothenic acid becomes effective.

It must be admitted that up to the present time knowledge regarding the fundamental role or roles of pantothenic acid is very scanty. Many effects of deficiencies on animals will be mentioned in later paragraphs but these are mostly, without doubt, secondary.

Dorfman, Berkman and Koser (127) have recently studied the effects of pantothenic acid on the metabolism of *Proteus morganii* (110). Oxygen uptake by deficient cells with pyruvate as substrate was greatly stimulated by pantothenic acid, even though no increase in cell numbers occurred, but no experiments were carried out using non-living systems, and the position occupied by pantothenic acid in the metabolism mechanism could

not be ascertained. The authors concluded that probably pantothenic acid has some role in connection with the oxidation of pyruvic acid. If it is directly concerned in this, it must play a dual role (and this is not impossible) because pantothenic acid is absolutely essential to the metabolism of many organisms which yield lactic acid as an end product of metabolism. It seems not improbable that when bacteria utilize lactic or pyruvic acid as a fuel, complex preliminary syntheses must precede their utilization, and that pantothenic acid enters into some of these processes.

The presumption, in view of the known functions of thiamin, nicotinamide and riboflavin, is that pantothenic acid fits into some enzyme system (or systems) which is essential to metabolism. What this enzyme system is or what these enzyme systems are is not known. There are some facts which suggest that pantothenic acid may be concerned with carbohydrate metabolism, but this is not certain. Wright (128) has recently shown that glucose administration to rabbits causes a lowering of the pantothenic acid content of the blood. This again strongly suggests a function in carbohydrate metabolism, but the effect may be indirect.

It may well be emphasized that the role played by pantothenic acid is without doubt a fundamental one, since it appears to be present in every living cell. In a complex organism it is essential to all types of cells and to the functioning of all kinds of tissues. It is not surprising, in view of this fact, that diverse pathological changes may result from its deficiency.

## 2. *Deficiencies and Requirements*

**General Discussion.**—Among the symptoms which have been fairly well established as connected with pantothenic acid deficiency in animals or fowls are the following: dermatitis; keratitis; adrenal hemorrhage, atrophy and necrosis; cortical fat depletion; "blood caked" whiskers; depigmentation of the hair (or feathers); failure to grow; loss of weight (adult); loss of appetite, emaciation; loss of coordination; loss of hair (alopecia); thymus involution; fatty livers; stomach and intestinal ulcers, diarrhea; heart damage; kidney damage; anemia; rapid respiratory rate; rapid heart rate; prostration or coma; sudden death; convulsions; gastrointestinal symptoms; loss of viability (eggs); paralysis; myelin degeneration, sciatic nerve and spinal cord damage; peripheral neuritis; sores about the mouth and nose; hemorrhages under the skin, severe oral lesions; abnormal cartilage (tibia); spinal curvature; increased appetite for salt.

It seems reasonable that only the lack of a substance, fundamental to cellular physiology in general, could cause such diverse symptoms. From



a study of the tissues of chicks deficient in pantothenic acid (129), it was concluded that every tissue became deficient, and presumably this would be true for any deficient animal. It seems that the particular part of the animal's mechanism, which appears to break down, depends upon the peculiarities of the species and the character of the examination made. If ways were available to carry out sufficiently delicate tests, every tissue in a deficient animal would presumably be found to be pathological. In individual species, particular tissues are susceptible or resistant, as the case may be, to pantothenic acid deficiency. The fact that in deficient mice the adrenal cortex may appear normal does not mean, of course, that pantothenic acid is non-essential for the adrenal cortex in the mouse, but rather that the adrenal cortex of the mouse has a more effective means of protecting itself against deficiency than the adrenal cortex of the rat.

A study of the data on the "B vitamins" in normal tissues (78) shows that in different species peculiarities in vitamin distribution in particular tissues exist, which may have a bearing on the question under consideration. Hog *muscle* (not hog tissues generally) is rich in thiamin and presumably might be resistant (or susceptible) to thiamin deficiency to a higher degree than other hog tissues. Beef *heart*, on the other hand, is considerably richer in thiamin than hog, rat or mouse heart, and this would be expected to have an effect on the resistance of this particular organ in this species to thiamin deficiency. Human muscle is richer in pantothenic acid than beef, hog, rat or mouse muscle (but not in other B vitamins), and one would expect this to have an effect on the ability of human muscle to resist pantothenic acid deficiency. It seems probable that the distribution of pantothenic acid (and other B vitamins) in the tissues of various species has an important bearing upon the question of how different animals react to deficiencies.

It will obviously be impossible in the available space to discuss all of the studies which have had to do with pantothenic acid deficiency. An attempt will be made to present the essential information regarding some of the more interesting and conclusive studies.

**Experiments with Fowls.**—The recognition of pantothenic acid as the "chick antidermatitis vitamin" has been discussed in an earlier section of this review. From the standpoint of the effects of pantothenic acid deficiencies on various animals discussed above, it becomes apparent how unsuitable the earlier name would have been for permanent retention, carrying with it the implication that the function of the vitamin is simply to prevent dermatitis, whereas in actuality all tissues are affected by its lack (129).

Phillips and Engel (130) have made a preliminary survey of the pathology of pantothenic acid deficiency in the chick. They found and described extensive spinal cord lesions, which were curable only by pantothenic acid. Sciatic nerve degeneration was observed to be associated with riboflavin deficiency. The conditions attributable to pantothenic acid deficiency included, aside from the spinal cord lesions, thymus involution, keratitis, dermatitis and fatty livers. The thymus involution seemed to occur when either pantothenic acid or riboflavin was deficient.

So far as the adult fowl is concerned Lepkovsky, Taylor, Jukes and Almquist (131) found the "filtrate factor" to have no apparent function in maintaining normal egg production or hatchability. The content of the eggs, however, was directly influenced by the diet. Bauernfeind and Norris (132, 20), however, found the antidermatitis vitamin to be essential for reproduction and hatchability, but not for egg production. Hens were on a diet supposedly free from the vitamins for 28 weeks, without developing any dermatitis and without any effect on egg production or mortality. In a later study from the same laboratory (119) after pure pantothenic acid became available, it has been shown conclusively that pantothenic acid is necessary for reproduction. On a basal diet even with added yeast filtrate and heated liver extract the hatchability of the eggs was zero. With suitable supplements, including pantothenic acid and unknown factors, the hatchability of the eggs could be brought up to 50-60%, but no diet lacking pantothenic acid could support reproduction. They concluded that pantothenic had some but not a great effect on egg production, and that its lack increased mortality. In seven weeks on the deficient diet, 80% of the hens developed a mild dermatitis on their lower shanks and feet.

The seemingly increasing importance of pantothenic acid for the mature fowl, as the result of more extended study, leads one to conclude that the diets used in the earlier studies contained appreciable amounts of undecomposed vitamin, and suggests the possibility that basal diets now in use may not be entirely free from it. Heating a diet in the dry condition may cause part of the vitamin to be "bound" in such a way that it is not extractable by ordinary procedures, and hence might not reveal its presence in a microbiological assay. Something of this sort happens to thiamin (133).

Snell, Aline, Couch and Pearson (134) studied quantitatively the effect of the diet of the hens on the pantothenic acid content of eggs. Eggs obtained from hens on a stock diet contained 17  $\gamma$  pantothenic acid per gram; from hens on a deficient diet (75% whole corn) eggs contained

3.6  $\gamma$  per gram. When extra pantothenic acid was fed the content became about double that on the stock diet. Snell and Quarles (135) found in connection with other studies that pantothenic acid, as might be expected, was not synthesized during incubation of eggs.

Taylor and co-workers (136) have found that increasing the pantothenic acid content of eggs by feeding the hens an extra amount or by injecting it directly into the incubating eggs modifies embryonic development. Hatchability was increased 15 to 30% over the controls, the hemoglobin content of the blood was increased 8 to 16%, and the heart size decreased as much as 17%.

The pantothenic acid requirement of chicks has been set by Jukes (137) at 1.4 mg. per 100 gm. of diet. According to a recent study by Bauernfeind (138) the requirement of single comb leghorn chicks is less than half of this, 600  $\gamma$  per 100 gm. of diet, for maximum growth. This low figure is difficult to accept as an unqualified optimal requirement in view of the extensive work of Jukes and the observation that chicks may develop dermatitis on a diet containing 75% whole corn (134). Corn has been found to contain 700–1000  $\gamma$  per 100 gm. (87, 88); accordingly 75% whole corn in the diet should furnish the optimal amount of pantothenic acid, and should by no means induce dermatitis.

Groody and Groody (139) recently have observed that pantothenic acid deficiency causes feather depigmentation in black chickens which is an interesting parallel with animal findings to be discussed later. They also observed that force-feeding chickens a diet low in pantothenic acid resulted in their death.

Lee and Hogan (140) have recently studied the requirements of the pigeon and conclude that "vitamin B<sub>5</sub>" of Williams and Waterman (16) while multiple in nature is primarily pantothenic acid. Their conclusion is in line with the experimental findings of Carter and O'Brien (141).

**Experiments with Rats.**—Because rats are a favorite animal for nutritional studies, the reports dealing with pantothenic acid in rat nutrition are too numerous and extensive to be discussed adequately and individually in a short space. We shall consider various of the pathological features resulting from pantothenic acid deficiency, which appear most important at the present time.

Morgan and Simms (142) were the first to note adrenal damage, excessive vascularity and later atrophy of the cortex, due to "filtrate factor" deficiency. This deficiency in rats has been studied by Daft and Sebrell (143, 144) and from the standpoint of pathology by Asihburn (145), and more recently by Supplee and co-workers (146). The direct connection

between pantothenic acid deficiency and the adrenal condition has been established in a number of additional laboratories (147, 148, 149).

It appears that pantothenic acid deficiency in rats damages the adrenal cortex seriously and that a number of the symptoms associated with the deficiency are due to loss of function of the adrenal cortex. The adrenal cortex appears to have numerous functions among which regulation of salt balance and water balance is prominent. It is very interesting that a low salt content of the diet favors graying due to pantothenic acid deficiency (150) and that pantothenic acid deficiency increases the appetite of rats for salt (146). Also it is interesting that water deprivation results in a production of "blood-caked whiskers" in rats (151, 152), a condition commonly associated with pantothenic acid deficiency, and that no combination of vitamins will cure the condition as long as the water is withheld (152). Chronic poisoning with zinc chloride precipitates a syndrome similar to pantothenic acid deficiency and its effects can be neutralized by pantothenic acid (153).

The anti-gray hair effect of pantothenic acid has been studied extensively. The graying of hair due to lack of filtrate factor, observed by Morgan and co-workers (24), has already been mentioned. Following or accompanying the announcements of György and co-workers (154, 155) that pantothenic acid administration would cure the gray-hair condition, there was a considerable amount of contrary evidence. Nielsen, Oleson and Elvehjem in a note (156) indicated that they had obtained a small amount of crystalline material with low pantothenic acid content which was effective for rats at a level of 15  $\gamma$  per day. Previous to this, workers in the same laboratory tested three pantothenic acid concentrates for anti-gray hair potency with negative results (157). Dimick and Lepp (158) gave evidence to indicate that the anti-gray hair factor is complex. R. R. Williams (159) found pantothenic acid to be of no value in protecting rats from gray hair, on the diets used, and Frost, Moore and Dann (160) found synthetic pantothenic acid ineffective whereas extracts containing comparatively little pantothenic acid were.

From these diverse results it is clear that the graying of hair is a complex process and that the condition may have more than one etiology, and be influenced by various factors. The effect of salt intake on graying (150) has already been mentioned. Cystine was found to decrease the time necessary for restoration of gray hair after pantothenic acid deficiency. Extracts of adrenals, pituitaries, thyroids or desoxycorticosterone were ineffective (161). Pyridoxin-deficient rats have a strong tendency to resist graying (162). Emerson and Evans (163) found pantothenic acid

to cure graying but not a stippling. Free (164) found graying in the rat to be due either to a vitamin lack or to a lack of minerals: iron, copper and manganese. Graying due to copper deficiency (165) was found by Elvehjem and co-workers (166) not to respond to pantothenic acid as did other graying.

As investigation has proceeded the importance of pantothenic acid as an anti-gray hair factor has increased. One of the most extensive investigations is that of Unna, Richards and Sampson (167) who not only found pantothenic acid effective, but found liver extract no better, on the basis of an equivalent amount of pantothenic acid. Their experiments indicated that inositol, *p*-aminobenzoic acid and biotin were ineffective either as substitutes for or supplements to pantothenic acid. On the basis of these experiments one would almost be tempted to justify designating pantothenic acid as *the* anti-gray hair vitamin. It is obvious, of course, that various vitamins of the B group, some of which may arise from bacterial action in the intestine, are important factors in maintaining healthy pigmented hair. The status of pantothenic acid as an anti-gray hair vitamin is strengthened by the finding of Wisconsin workers (166) that it prevents graying induced by a heated grain diet, and that *p*-aminobenzoic acid was ineffective. The work of Martin (185) with mice, to be discussed later, also stresses the importance of pantothenic acid as an anti-gray hair factor. The depigmentation of feathers of fowls due to pantothenic acid deficiency has been mentioned (139).

Other conditions which have repeatedly been observed in connection with pantothenic acid deficiencies in rats are dermatitis (168, 149, 169) sores about the mouth and nose (168, 149) so called "blood-caked whiskers" (170, 149), in which the deposit is porphyrin derived from the Harderian gland (171). The possible connection between this latter condition and the adrenal cortex has been mentioned. Dermatitis is, of course, an extremely indefinite condition and may have many causes. The fact that pyridoxin or pantothenic acid deficiencies may cause lesions which appear to be about the same (169) is not entirely surprising because both vitamins are necessary for a healthy skin, and there is no apparent reason why certain skin areas might not be susceptible to different deficiencies. Alopecia is another sign of pathological skin which has been observed in pantothenic acid deficiencies (168, 149).

Miscellaneous conditions which have been connected with pantothenic acid deficiencies in rats are: hemorrhages under the skin (149), kidney and heart damage (146), thinness of epiphyseal cartilage of the tibia (145) and sudden death (146).

It appears probable that with different levels of deficiencies, different lesions would appear. On a diet completely free from pantothenic acid probably few lesions would appear before death, as compared with those which might develop on a diet in which some pantothenic acid is supplied. The different types of syndromes which appear may be due largely to the degrees with which deficiency exists.

A few miscellaneous facts regarding the functioning of pantothenic acid in rat nutrition are worthy of note. Supplee and co-workers (172) have found that during the assimilation of food there is mobilization of riboflavin to the liver, and that pantothenic acid has a direct and specific function in connection with the process. Drill and Overman (173) have found that the pantothenic acid (also pyridoxin and thiamin) requirements of rats are increased during thyroid feeding. Elvehjem (174) has indicated that pantothenic acid may promote the intestinal production of other B vitamins in the rat. Taylor and co-workers (175) find increased litter size in rats, to result from pantothenic acid administration.

There is fairly good agreement among various workers who have studied the problem that the pantothenic acid requirement of the rat is about 80  $\gamma$  to 100  $\gamma$  per day. Unna (149) made a special study of the problem and concluded that about 80  $\gamma$  per day yields optimal growth. About 100  $\gamma$  per day is enough to support reproduction (176). Recently Unna and Richards (177) have studied the problem of the requirements of rats of different ages, and found that the requirement, unlike that of thiamin, decreases markedly with age. They suggest that the higher requirement during youth may be due to the association of pantothenic acid with the process of building new tissue. It may be that the decreased nutritional requirement with age may be associated with greater bacterial production in the cecum of the adult rat (178) rather than a decrease in the requirements of the adult tissues.

**Experiments with Mice.**—The study of the functions of pantothenic acid in mice has been complicated by the question of the exact status of inositol as a vitamin for mice, and by the existence of unknown vitamins necessary for mice and present in extracts.

Graying of hair in mice has not been studied as extensively as the same phenomenon in rats. György and Poling (155) found that graying could be induced in mice by pantothenic acid deficiency and cured by its administration. Like graying in rats it could not be cured completely for the maintenance of a normal pelt indefinitely by pantothenic acid alone; biotin also appeared to be necessary.

Alopecia has been observed a number of times in mice on diets deficient

in pantothenic acid (155, 179, 180, 181, 182). In the early experiments of Norris and Hauschildt (179) extensive alopecia was observed, for which the lack of some factor other than the ones then available was responsible. Later Martin (180) observed the same symptoms on the same diet and found that 150  $\gamma$  of pantothenic acid per day caused immediate curative response. The effect of pantothenic acid on alopecia in mice is complicated by the question of the effectiveness of inositol, discovered to be a vitamin for mice by Woolley (183). His studies indicate that while pantothenic acid may have a curative effect on alopecia, its effect is indirect, by inducing intestinal synthesis of inositol (184). The situation from the dietary standpoint is complex, because he found that a deficiency of inositol could develop even when inositol is in the diet, provided pantothenic acid is absent.

Martin (185) finds that thiamin, riboflavin, pyridoxin, niacin, pantothenic acid and choline, added to a basal B complex-free diet, renders it adequate for mice and just as complete as one containing inositol and *p*-aminobenzoic acid in addition. Addition of either inositol or *p*-aminobenzoic acid precipitates a syndrome which can be overcome only by the addition of the other. He explains the finding of Ansbacher (186) with regard to the efficacy of *p*-aminobenzoic acid as an anti-gray hair factor, on the basis that inositol was introduced into the diet used, and only because of its presence was the striking effect of *p*-aminobenzoic acid observed. He also indicates that only when *p*-aminobenzoic acid is in the diet are the effects of added inositol readily and uniformly observed.

Some of the general symptoms and pathological changes resulting from pantothenic acid deficiency in mice have been described by two groups of workers (181, 182, 187). Sandza and Cerecedo (181) mention spinal curvature, serous exudate around the eyes, a kicking twitch of the hind legs in addition to alopecia. Lippincott and Morris (187) found myelin degeneration in sciatic nerves and spinal cord (accompanying paralysis of the hind quarters (182)), and hyperkeratotic, atrophic and desquamative dermatosis. The adrenal glands remained normal, in contrast to the observations on rats. Adult mice lost weight (182).

Morris and Lippincott (188) found that pantothenic acid deficiency in mice definitely lowers the rate of growth of mammary carcinomas, but simultaneously causes a severe interference with the host's nutrition. The average daily food intake of the mice was about the same before and after the administration of pantothenic acid. Lewisohn and co-workers (189) found the injections of yeast extracts to prevent tumor growth in 20% of implanted mice. This percentage was about doubled when panto-

thenic acid was administered with the yeast extract, and about tripled when riboflavin was used. Taylor and co-workers (175) found increased viability of eggs (increased litter size) could be induced in mice by pantothenic acid administration.

Sandza and Cerecedo (181) concluded that the pantothenic acid requirement of mice is about 30  $\gamma$  per day. Morris and Lippincott (182) found 23–29  $\gamma$  per day nearly as effective as over 200  $\gamma$  per day, so there is substantial agreement on this point so far.

**Experiments with Dogs.**—A number of preliminary studies (189, 25, 190) had indicated the probable importance of pantothenic acid in the nutrition of dogs, before the more extensive report dealing directly with this subject was published by Schaefer, McKibbin and Elvehjem (192). This report gives in very satisfactory form about all that is known at the present time regarding pantothenic acid deficiency in dogs.

One of the characteristics of the deficiency is the suddenness with which the animals may fail. Often they eat normally up to the day of the onset, and must be observed frequently if treatment to save their lives is to be administered. There is sudden prostration or coma; usually, but not always, rapid respiration and heart rate; and gastro-intestinal symptoms. In some cases, treatment of a severe deficiency brought the animal back to health even though weeks were required. In other cases the dogs died in spite of treatment. Evidently pantothenic acid deficiency may result in severe damage which is difficult or impossible to repair.

The condition of coma may be related to the hypoglycemia which was observed in deficient dogs, and which disappeared when the deficiency was treated with pantothenic acid. The respiratory and gastro-intestinal symptoms and those involving the skeletal muscles (convulsions) may have had their origin in nervous lesions.

Necropsies were performed on all the dogs which died of deficiencies, but no histological examination of the tissues was recorded. All animals had light-colored mottled livers with very high fat content. The presence of fatty livers and hypoglycemia (and presumably low liver glycogen) on a diet containing 66% sucrose was pointed out as noteworthy, and suggestive of a fundamental impairment of carbohydrate metabolism. Mottled thymuses suggestive of hemorrhagic degeneration were generally observed. The kidneys were dark red in color and showed microscopic evidence of hemorrhage in cortex and medulla. Gastritis and severe enteritis were common and intussusception in the pyloric region and the lower ileum was observed.

The adrenal glands appeared macroscopically normal with the exception



of one out of six which was enlarged. The fact that the blood chlorides were about 20% low in the deficient dogs, and rose after treatment, suggested the possibility of impairment of the adrenals, which, however, did not in general appear macroscopically.

No observations were made in this report regarding the graying of the hair of the dogs except that graying had been observed in dogs which had abundance of pantothenic acid in the diet. Such graying did not necessarily have a nutritional origin.

The requirement of young puppies was estimated to be about 100  $\gamma$  of calcium pantothenate per kilo of body weight per day. That of adult dogs is thought to be considerably less.

Three miscellaneous observations are worthy of note: Russell and Nasset (192a) found that carbohydrate digestion and absorption were increased 51% and 37%, respectively, when 2 mg. per day of calcium pantothenate was given to dogs on a Purina Chow diet. Other synthetic vitamins failed to have this effect. Morgan (193) in a preliminary report gave some evidence to indicate that dogs deficient in "filtrate factors"—nicotinic acid, pantothenic acid and unknown factors—were harmed rather than benefited by administration of nicotinic acid or pantothenic acid alone, and suggested the undesirability of an imbalance. Silber and Unna (194) studied urinary excretions of pantothenic acid by dogs and found that pantothenic administration had no effect on the riboflavin level of the blood. This is in contrast to the findings of Spies and co-workers (202) on humans to be mentioned later.

**Experiments with Other Animals.**—Most of the available published information regarding pantothenic acid in the nutrition of hogs is that from Hughes and co-workers (195, 196). Deficiency in this animal leads to poor growth (in pigs); rough dry coat; emaciation; loss of hair in some cases; congested, hemorrhagic and ulcerated areas in the stomach and large intestine particularly, and lack of co-ordination (goose stepping with the hind legs). As in the case of the dog the gastro-intestinal symptoms are prominent.

The requirement of growing pigs is thought to be 7.8–11.8 mg. daily per 100 lbs. of animal. Calculated on the same basis, this requirement is over twice that indicated for the growing pup (192).

It has been known for years that various B vitamins are produced in the rumen of cattle. Three studies have recently been made, dealing with the production of pantothenic acid in the rumen of cattle and sheep.

Wegner, Booth, Elvehjem and Hart (197) showed that pantothenic acid and other B vitamins are synthesized in a cow's rumen when the cow is

fed a diet deficient in B vitamins. When thiamin was added to the diet it seemed to stimulate the bacterial synthesis of other B vitamins.

Later the same authors (198) studied the synthesis of B vitamins including pantothenic acid in a heifer fed a natural diet of silage, hay and grain. The pantothenic acid content of the ration was about 10  $\gamma$  per gram, whereas the rumen contents, obtained by fistula, were on the average nearly three times as rich.

McElroy and Goss (199) fed a diet to sheep and cows, which contained less than 2.8  $\gamma$  of pantothenic acid per gram. The contents of the sheep rumen and reticulum contained 70  $\gamma$  pantothenic acid per gram, and were therefore about 25 times as rich as the feed. In the cow the rumen contents were 20 to 30 times as rich in pantothenic acid as the feed. It is possibly significant that the pantothenic acid content of the rumen contents, according to these workers (using the chick assay method), was in general over twice as large as that given by the Wisconsin workers who used a microbiological assay. Phillips and co-workers (200) found that diarrhea in young calves could be prevented by administration of vitamins A and B. They concluded that nicotinic acid and pantothenic acid may be the effective members of the B family in this condition.

Chapman and Harris (201) found that monkeys maintained on diets deficient in pantothenic acid, and probably some other B vitamins, developed severe oral lesions, "marked general symptomatology" and showed short survival times. The oral lesions were accompanied by an increase in fusospirochetal flora.

**Human Experiments and Deductions.**—Spies and co-workers (202) were the first to present direct evidence to indicate that pantothenic acid has a function in human nutrition. They found that concentration of pantothenic acid in the blood of malnourished patients was substantially low compared with normal individuals. Injection of pantothenic acid produced a temporary rise in the pantothenic acid level of the blood, and also a 20–30% rise in the blood riboflavin level both in normal and malnourished individuals. These findings are of interest in connection with the more recent observations of Supplee and co-workers (172) on rats and Silber and Unna (194) on dogs. Pearson (114), Wright and Wright (115), Gordon (113) and Pelczar and Porter (112) have studied pantothenic acid excretion in man with substantial agreement as to normal excretion. "Normal" individuals excrete on the average something over 3 mg. per day. Gordon reports that the "test dose procedure" is not applicable as a diagnostic measure of human pantothenic acid deficiency.

Gordon (113) has recently reviewed the available evidence regarding

pantothenic acid deficiencies in man, and cites five cases of peripheral neuritis, one case of Korsakoff's syndrome in a male alcoholic with severe peripheral neuritis, two cases of delirium tremens (203), all of which responded promptly and markedly to pantothenic acid administration when other B vitamins had failed to elicit a response. In some cases there might be a question whether the beneficial response was not due to a delayed effect of vitamins previously administered.

He also indicated the possibility of non-tuberculous Addison's disease being due wholly or in part to pantothenic acid deficiency. In private correspondence he has cited one case from which autopsy sections of the adrenals showed marked hemorrhage and necrosis, and which he feels positive was a case of pantothenic acid deficiency.

The fact that the "anti-neuritic" properties of thiamin have been seriously questioned, is mentioned and the growing opinion that "nutritional neuropathy" is caused by multiple deficiency. Pantothenic acid may prove to be one of effective vitamins in this connection.

The amount of information regarding human pantothenic acid deficiencies and their treatment is meager, but the various lines of evidence with respect to animals suggest very interesting possibilities. The writer has personal knowledge of one case of a nurse, who gave evidence of having received benefit particularly on the mental side (especially memory) as a result of medication with pantothenic acid for a considerable period. This observation is suggestive because Gordon reported a "rapid clearing of the mental state" of one of his cases.

The writer is not aware of any significant report on the status of pantothenic acid as an anti-gray hair vitamin for humans, aside from one by Punnett and Bader published in a popular magazine (*Good Housekeeping*, September, 1941, and September, 1942) in which a fair proportion of the individuals of various ages are reported to be benefited. If these findings are real they suggest that mild pantothenic acid deficiencies may be common, and that gray hair in some cases may be an outward manifestation of a mild pathological state in various tissues.

The human requirement of pantothenic acid is not known with any certainty. Gordon, on the basis of a comparison of riboflavin requirement and excretion, with pantothenic excretion arrived at the "entirely speculative" figure of 9-11 mg. per day.

Independently and in ignorance of this figure, Williams (204) arrived at practically the same value by an entirely different means. Various natural foods and food mixtures were assayed for pantothenic acid (and other B vitamins) including human and cow's milk, and the results showed a striking uniformity in that about 10-12 mg. of pantothenic acid was invariably associated with 2500 calories of what we have reason to believe are suitable foods. From these findings it may be deduced that 10-12 mg.

of pantothenic acid per day (or per 2500 calories of food) is probably a perfectly safe level.

The subject of the human pantothenic acid requirements is, of course, in need of much study from several angles. Particularly since the needs of young animals seem to be greater than those of adult animals, the problem of the needs of children requires investigation. It may reasonably be supposed on the basis of present information that if human adult deficiencies exist (and there is some evidence that they do), deficiencies among children are more serious and widespread than those among adults.

### 3. Pharmacology

A noteworthy fact regarding pantothenic acid, which is shared to a greater or lesser degree by other vitamins, is that while it is essential to life, and detectable in extremely minute amounts, administration of it to a normal animal is practically without effect. Human beings also fail to give any response so far as blood pressure, pulse, temperature or respiration is concerned when about 10 times the normal daily intake (100 mg.) is injected (202). This suggests the probability that pantothenic acid in order to be effective must be built into tissue constituents; and that this is a relatively slow process and one which takes place only to an extent demanded by the needs of the organism.

Unna and Greslin (205, 206) have made a thorough study of its effects on animals. They found that a 10% solution could be instilled into the conjunctival sac, or injected (1 cc.) into rabbits without irritation or inflammation. The lethal dose is in terms of *grams* per kilo of body weight, in mice and rats.

Monkeys were fed one gram of calcium pantothenate daily for six months without any untoward effects or pathological changes. This is probably about 500 times their ordinary intake. Dogs and rats survived without damage daily administration for the same period of from 500 to 2000 times their daily requirement.

### 4. Functioning in Miscellaneous Organisms

It is well known that pantothenic acid is effective as a yeast growth substance and that its discovery and characterization came about for this reason (1). Unfortunately the term *yeast* is often used in a careless manner and sweeping and unwarranted statements are made on the basis of observations on one strain under a particular set of conditions. The term *Saccharomyces cerevisiae* while seeming to have a definite meaning is little better than *yeast* because it includes many strains of diverse behavior. This subject has been reviewed elsewhere (14). While pantothenic acid is stimulative of all strains tested, the differences in the responses were early noted (30).

The growth responses of yeast are complex because different strains do not have the same synthetic abilities for the various known nutrilites, thiamin,  $\beta$ -alanine, pantothenic acid, inositol, pyridoxin, biotin and folic

acid (207); and the amino acids (208). Furthermore, the synthetic powers of yeasts do not remain unchanged in all respects for an indefinite period (83). For these and other reasons the study of pantothenic acid in its relation to yeast physiology constitutes a difficult field of study.

The production of pantothenic acid by yeasts has been studied (82) and attempts to ascertain its fundamental role have been made (124, 125, 126), without coming to definite conclusions except that its "binding" by the yeast is preliminary to its utilization.

Green plants (and plant tissues, 125) may be stimulated by minute doses of pantothenic acid. This has been observed in the case of the liverwort, *Ricciocarpus natans* (209), alfalfa seedlings grown under sterile conditions (80) and pea embryos (210). The mobilization of pantothenic acid in a sprouting potato was early observed (209).

Elliott (211) observed the stimulating effect of a pantothenic acid concentrate on certain protozoa. The one species tested was found to be rich in pantothenic acid (98) as well as certain other B vitamins. Whether it is required by various protozoa is not known (212, 224).

The production of pantothenic acid by *Aspergillus niger* was early observed (1), and its stimulative effect on other molds (182, 213) has been studied. An extremely interesting finding is that of Beadle and Tatum (214) who were able to alter the genes in a mold, so that it became dependent upon an outside source of pantothenic acid. The observation that in one mold *any one* of several nutrilites had by itself the ability to promote growth, whereas growth was lacking in the absence of all (81), is worthy of note.

The relationship of pantothenic acid to various bacteria has been mentioned numerous times in this review. The requirement for pantothenic acid is shown by numerous lactic acid bacteria and some propionic acid bacteria as shown by Snell, Strong and Peterson (6), and others (215, 216). Among the other bacteria which require it as a nutrilitic, or for which it is effective, are: diphtheria bacilli (67, 217), hemolytic streptococci (61, 218, 62), *Proteus morganii* (110), certain members of the *Pasteurella* group (219), pneumococci (220, 221), and certain non-sporulating anaerobes (222). Bacteria which do not require pantothenic acid produce it, retain it in the cells and release it into the culture medium (100). Microbiological methods for pantothenic acid assay using bacteria have been discussed (105, 91, 111, 35, 11). A study of the function of pantothenic acid in the metabolism of *Proteus morganii* has been discussed. Its production in the intestines of animals (174, 178, 223) and in the rumen of cattle and sheep (197, 198, 199) has been studied.

Comparatively little attention has been given to the study of insect nutrition using pure chemicals. Trager (224) has reviewed this field. Subbarow and Trager (225) have found mosquito larvae to require pantothenic acid for development. Insects are a comparatively rich source of pantothenic acid and certain other B vitamins (98). Their nutrition is complex and probably pantothenic acid is a general nutritional requirement for larvae. The high pantothenic acid content of "royal jelly" (104) is probably not accidental, and suggests important functions in the nutrition of bee larvae.

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# THE CHEMISTRY AND BIOCHEMISTRY OF BIOTIN\*

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## I. Introduction

In recent years a great number of bacterial growth factors have been shown to play an important role in animal nutrition, and the close relationships between growth factors on the one hand and the vitamins on the other have become well recognized. One of the more recent developments in this field was the discovery by du Vigneaud, Melville, György and Rose (1) of the identity of biotin, a bacterial growth factor, with vitamin H, the curative factor for egg white injury. This finding demonstrated the general importance of biotin in the metabolism of the cell and was a mile-

\* Presented before the Seminar in Organic Chemistry, Fordham University, December 16, 1942.

stone in the development of the knowledge of the mechanism of egg white injury.

It is not intended to present a complete review of the literature on biotin, but rather to select some high lights of the important developments which have influenced the knowledge of the chemistry and the biochemistry of this substance.

Biotin was isolated as the crystalline methyl ester in 1936 by Kögl and Tönnis (2) and was identified in 1940 (1) with the curative factor for egg white injury. As the developments preceding the isolation of biotin have been reviewed extensively elsewhere (2, 3), they shall not be further discussed. However, egg white injury\* provides the background for numerous biological as well as biochemical studies, and therefore justifies a more detailed treatment.

Rats which are fed a well-balanced diet, to which has been added a large portion of dried egg white as the source of protein, develop a severe dermatitis accompanied by nervous disorders. In the later stages of the disease the rats take a very typical kangaroo-like position due to a paralysis of the hind legs, and die if the condition is not relieved (6). The toxic properties of egg white are destroyed by heat, digestion with pepsin, or incubation with hydrochloric acid. There is present in foodstuffs an organic substance which is able to neutralize the toxic effects of the egg white when given as a supplement to the otherwise disease-producing diet. This substance has been called "protective factor X" by Boas (6), the "factor protective against egg white injury" by Lease and Parsons (7) or "Vitamin H" by György (8).

Using a curative rat assay for the estimation of vitamin H activity, György (9, 10, 11) published extensive studies on the distribution as well as the purification of this substance. He found that the factor is widely distributed in nature and that liver and yeast are especially rich sources. Hydrolysis of the tissues was necessary to liberate the vitamin which is present in a nondialyzable form. Appreciable concentration could be effected by the use of adsorption as well as precipitation steps, and from the electrophoretic behavior it was concluded that vitamin H is an amphoteric substance, having an isoelectric point of  $pH$  3-3.5.

The steps used by Kögl for the concentration of biotin from egg yolks were similar to those employed by György, and the parallelism in distribution, as well as in chemical behavior, suggested to György, Melville, Burk and du Vigneaud (12) that vitamin H might be identical with biotin. Consequently, a vitamin H concentrate was subjected to electrodialysis and it was found that the distribution of the vitamin H and biotin activities in the different cells of the dialyzer showed striking similarity.

A sample of Kögl's biotin methyl ester, when tested for vitamin H activity, was found to be extremely potent in protecting rats against the

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\* A detailed discussion of egg white injury is presented in references 4 and 5.

injurious effects of egg white (1), thus establishing the identity of vitamin H with biotin. These developments were followed by the isolation of biotin from liver (13, 14) and as more of the crystalline material became available, the way was cleared for an attack on the chemical structure of biotin.

## II. Biotin Standards

In all of the work preceding the isolation of pure biotin, standards had to be set up to express the potency of a given preparation. Kögl (2), using his *Saccharomyces* unit (S. U.), which he defined as the amount of biotin producing 100% increase in cell growth of a strain of yeast (Rasse M) under standard conditions, found that crystalline biotin methyl ester had a potency of 25,000,000 S. U. per milligram. György (9), on the other hand, described a curative vitamin H rat unit which was defined as the daily dose of vitamin H that would cure a rat of egg white injury in four weeks. Following the establishment of the identity of biotin with vitamin H, a biotin concentrate from liver containing 1000 vitamin H units per cc., as assayed by the rat method, was employed as the biotin standard. Consequently the biotin activities of the various fractions obtained during the isolation of biotin from liver (14) have been expressed in terms of vitamin H units, despite the fact that the yeast growth method was used as the assay procedure. The activity of pure biotin methyl ester was found to correspond to 27,000 ( $\pm 10\%$ ) vitamin H units per mg., which means that a daily dose of  $3.7 \times 10^{-5}$  milligrams of this material can cure a rat of egg white injury.

Once the crystalline vitamin was at hand, all further standardizations were made on the basis of the pure substance, and the content of a given preparation could be expressed in actual amounts of biotin.

## III. Assay Procedures

The only available accurate procedures for the estimation of biotin are the microbiological growth methods, which have been developed with great success in recent years.

The first microbiological procedure for the estimation of biotin was the one described by Kögl (2) and employed in his work on the concentration of biotin from egg yolks. In this method a strain of yeast "Rasse M" was used as the test organism, and the growth response of a heavy inoculum was determined with a photoelectric colorimeter after an incubation time of five hours. Snell, Eakin and Williams (15) have developed an excellent yeast growth method for biotin assay using a strain of *Saccharomyces cerevisiae* isolated from Fleischmann's bakers' yeast. The growth response of a small



inoculum is determined with a special thermoelectric turbidimeter (16) or a suitable photoelectric colorimeter after sixteen hours of incubation. As the method has been described in great detail (17), no further comment is necessary here. Numerous other procedures for the quantitative estimation of biotin have been described, and several of them merit special mention.

Lampen, Kline and Peterson (18) suggested the organism *Clostridium butylicum* as a test organism for a turbidimetric method of biotin assay. This organism is especially suitable since it requires only biotin as an accessory growth factor (19).

Another assay method has been proposed by Shull, Hutchings and Peterson (20) using the increase in titratable acidity produced by *Lactobacillus casei*  $\epsilon$ ,\* which under certain conditions is a measure of the biotin content of the medium. This procedure offers the advantage of being applicable to turbid and colored solutions where the turbidimetric methods cannot be employed.

West and Woglom (21) have given a detailed description of their assay procedure in which they measure the influence of biotin on the increase in turbidity of cultures of *Rhizobium trifolii*.\*\*

It seems that, at present, the yeast growth method of Snell, Eakin and Williams (15, 17) represents the most satisfactory technique for the estimation of biotin.

In conclusion, it might be mentioned that animal assay procedures have likewise been suggested to determine biotin in natural materials. György (9), for example, used the curative rat method for the estimation of vitamin H, prior to the establishment of its identity with biotin. Other investigators (26, 27) have also suggested the use of chicks as test animals.

#### IV. Isolation of Biotin

Biotin methyl ester was isolated from egg yolks for the first time in 1936 by Kögl and Tönnis (2). In a tedious fractionation procedure, using sixteen different steps, they were able to obtain 1.1 mg. of pure biotin methyl ester from 250 kg. of dried Chinese egg yolks. This corresponds to a recovery of 1.8% of the biotin present in the starting material. By modification of the original procedure, however, the yield of 1.8% could recently be raised to 20% (28).

The method employed by Kögl is briefly as follows:

Active extracts were prepared from egg yolks by extraction with hot water,† and from these extracts, by skillful use of different precipitation as well as adsorption techniques, there was obtained a highly active concentrate. This concentrate was then subjected to an esterification procedure yielding a chloroform-soluble basic ester fraction from which the crystalline biotin methyl ester was isolated by fractional distillation. In attempts to isolate the curative factor for egg white injury (Vitamin H) (10), very similar

\* This organism is unable to utilize biotin methyl ester (20).

\*\* Biotin has been shown to be identical with "Coenzyme R," a growth and respiration factor for rhizobia (22, 23, 24, 25).

† Egg yolks contain biotin in a bound form from which it can be liberated by treatment with hot water (29).

steps were employed to prepare active concentrates, and the knowledge gained from these earlier studies was extremely helpful for the more recent work by du Vigneaud, *et al.*, which led to the isolation of biotin from liver (14) and milk (30).

The starting material for the isolation of biotin from liver was a liver concentrate which had been prepared according to the procedure of György (10), and which assayed 34 vitamin H units per mg. This material was esterified, and the crude ester fractions assaying 50 vitamin H units per mg., were subjected to chromatographic adsorption procedures. The active ester fraction was dissolved in chloroform and filtered through a column of activated aluminum oxide. The column was then eluted with different solvents, and the weight and activity of each eluate were determined separately. Table I illustrates such an experiment.

TABLE I  
DISTRIBUTION OF BIOTIN METHYL ESTER\* IN AN ALUMINUM OXIDE CHROMATOGRAPH

Column eluted with		Eluted fraction		
		Weight in mg.	Total activity in vitamin H units	Vitamin H units per mg. solids
Chloroform	Cc. 300	15,000	147,000	9.8
Acetone	300	805	1,000,000	1242
Acetone 90% + methanol 10%	50	21	45,000	2140
Acetone 90% + methanol 10%	50	76	145,000	1910
Acetone 90% + methanol 10%	50	256	310,000	1210
Acetone 90% + methanol 10%	50	102	45,000	440

\* Starting material was a crude ester fraction containing 50 vitamin H units per mg. of solids.

TABLE II  
ACTIVITY AND PURITY OF DIFFERENT FRACTIONS OBTAINED DURING THE CONCENTRATION OF BIOTIN FROM LIVER

Fraction	Activity, vitamin H units per mg.	Purity, %
Crude vitamin H concentrate from liver	34	0.13
Crude ester fraction	50	0.18
Material from first chromatograph	2000	7.4
Material from second chromatograph	5000	18.5
Pure biotin methyl ester	27,000	100.0

As may be seen from the table, the most active fractions were eluted with acetone or a mixture of 90% acetone and 10% methanol. A concentration from 50 vitamin H units per mg. to 2000 vitamin H units per mg. was achieved in one step.

A repetition of the same chromatographic procedure yielded fractions containing 5000 vitamin H units per mg., from which, after a second esterification step, crystalline biotin methyl ester was obtained. Table II illustrates the degree of purification which was ef-

fects in each step of the fractionation procedure, and indicates the purities of the different fractions.

Expressed in terms of vitamin H units, the crystalline ester had a potency of 27,000 vitamin H units per mg.

The ester was subjected to several recrystallizations and sublimations *in vacuo* without changing its biological activity or melting point. Kögl initially reported a melting point of 149° for his crystalline material, whereas du Vigneaud, *et al.*, consistently obtained fractions melting at 166–167°. In a later paper (28) Kögl reported material melting at 161.5°.

In view of the fact that identical degradation products have been obtained from the “egg” as well as the “liver” and “milk” biotin, there can be no doubt that biotin from these different sources is one and the same substance.

## V. Chemistry of Biotin

### 1. Properties and Elementary Composition

Biotin methyl ester crystallizes from a mixture of methanol and ether in elongated plates. Mixtures of chloroform and petrol ether, as well as mesityl oxide, have likewise been proposed as solvents for the crystallization. The most effective purification procedure, especially to free the crude ester from colored impurities, is sublimation *in vacuo*. The ester is very soluble in methyl alcohol, ethyl alcohol and chloroform; it is sparingly soluble in benzene and insoluble in ether, petrol-ether or water. Preparations of the ester which have been isolated from egg yolks (2, 28) were found to contain oily impurities which could be removed only with the greatest difficulty. No such impurities were observed when liver or milk was employed as the starting material. Biotin methyl ester has an optical activity of  $[\alpha]_D^{22} = +57^\circ$  in chloroform.

Biotin was prepared for the first time by du Vigneaud, *et al.* (31), by saponification of the ester with cold alkali. It crystallizes from water in needles melting at 230–232° with decomposition, and its rotation in 0.1 *N* sodium hydroxide is  $[\alpha]_D^{22} = +92^\circ$ . As measured by the yeast growth method the physiological activity of biotin, on a molecular basis, was identical with that of the methyl ester.

The correct empirical formula,  $C_{11}H_{18}O_3N_2S$ , for biotin methyl ester was reported in 1937 by Kögl (32) and confirmed by du Vigneaud, *et al.* (14). Spectroscopic studies which were performed on biotin methyl ester (28, 31) indicated no specific absorption bands in the range from 2200 to 6000 Å.

## 2. Functional Groups

The first steps taken toward the elucidation of the molecular structure of biotin were studies to determine the nature of the functional groups. One methoxyl group was found to be present in biotin methyl ester. The ester was easily saponified to form biotin when treated with dilute alkali in the cold (31). Biotin,  $C_{10}H_{16}O_3N_2S$ , in contrast to the methyl ester, did not liberate volatile iodides when treated with HI and consequently does not contain  $-OCH_3$ ,  $-NCH_3$  or  $-SCH_3$  groups. The titration curve of biotin, as shown in Fig. 1, closely resembled the titration curve of a monocarboxylic acid.

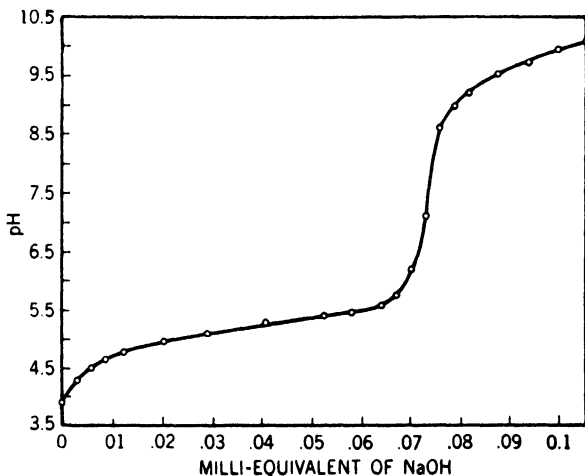


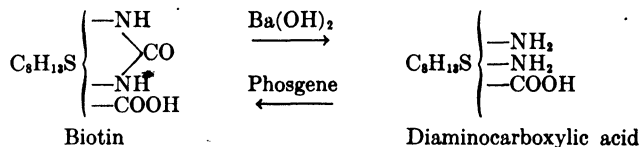
Fig. 1.—Electrometric titration curve of biotin.

Biotin crystallized even from strong HCl solutions as the free acid, and did not form a hydrochloride. These results were conclusive evidence that biotin is a monocarboxylic acid exhibiting extremely weak basic properties. No inactivation occurred when the substance was treated with ninhydrin, and no nitrogen was produced when biotin was subjected to the Van Slyke amino nitrogen procedure.\* These results show that biotin contains neither an amino group nor basic ring nitrogen atoms. Information as to the nature of the nitrogen atoms was obtained in the course of studies of the hydrolytic cleavage of the molecule. When biotin was subjected to

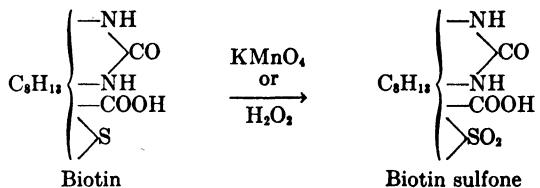
\* Snell, *et al.* (15), found that biotin is inactivated by treatment with nitrous acid. This inactivation is probably due to the formation of a nitroso compound.

a drastic hydrolysis with hydrochloric acid (28) or barium hydroxide (33), an optically active diaminocarboxylic acid,  $C_9H_{16}O_2N_2S$ , was obtained which was found to contain two primary amino groups by the Van Slyke nitrous acid method.

The diaminocarboxylic acid was characterized by the preparation of several derivatives. These included the dibenzoate, the diacetate, the sulfate, the dihydrochloride and the dipicolonate. The formation of the strongly basic diaminocarboxylic acid,  $C_9H_{16}O_2N_2S$ , from the weakly basic biotin,  $C_{10}H_{16}O_5N_2S$ , with the replacement of one CO group by two hydrogen atoms, pointed to a cyclic urea structure. The urea structure was established definitely when it was found that biotin was resynthesized from the diaminocarboxylic acid when it was treated with phosgene (34).



As mentioned earlier, biotin did not liberate volatile iodides when it was treated with HI. No indication of the presence of labile sulfur was obtained. It likewise had been shown (2, 35) that the compound does not contain an ethylenic linkage. Titration experiments with cold potassium permanganate, however, revealed an uptake of two atoms of oxygen with the formation of a new crystalline compound, biotin sulfone (36). Biotin sulfone,  $C_{10}H_{16}O_5N_2S$ , was likewise obtained in excellent yield when biotin was oxidized with hydrogen peroxide in glacial acetic acid (33).

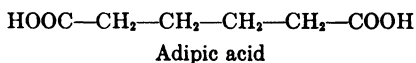


The formation of a sulfone established the thioether nature of the sulfur atom, and it was, therefore, concluded that biotin is a monocarboxylic acid containing a cyclic urea structure with the sulfur atom in thioether linkage.

### 3. The Nature of the Side Chain

With the knowledge of the nature of the functional groups at hand, the way was cleared for further degradation studies.

A great deal of information on the structure of biotin was obtained during studies of the oxidative breakdown of the diaminocarboxylic acid. Oxidation of the diaminocarboxylic acid with nitric acid or alkaline permanganate resulted in the formation of adipic acid,  $C_6H_{10}O_4$  (37, 38).



The isolation of this straight chain, dicarboxylic acid, containing 6 of the 9 carbon atoms of the diaminocarboxylic acid, reduced to a great extent the number of possible structures that could be assigned to biotin. These could be further reduced by establishing in the following way the identity of one of the carboxyl groups of the adipic acid with the original carboxyl group of biotin (38). A triamine (V) was prepared from biotin methyl ester by means of the Curtius degradation, thus replacing the carboxyl group of the diaminocarboxylic acid by an amino group.

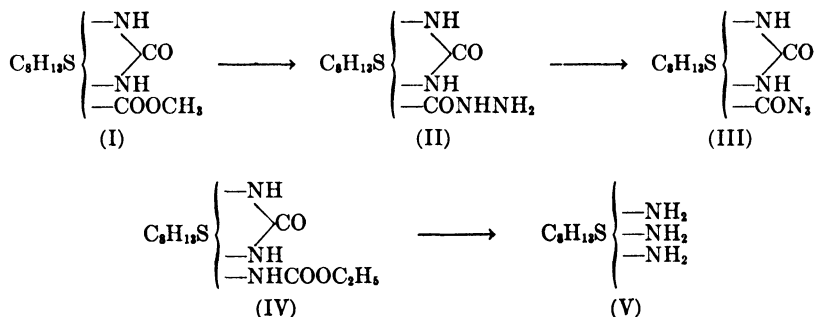


Fig. 2.

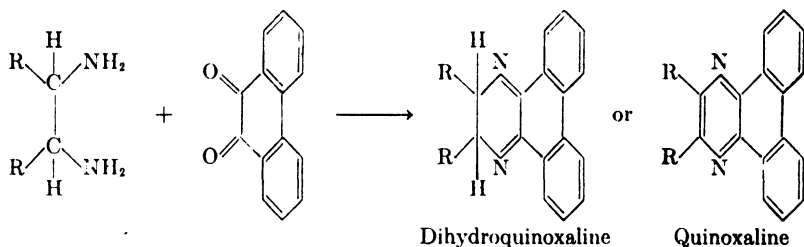
Biotin methyl ester (I) was converted into biotin hydrazide (II) by treatment with hydrazine hydrate, and the corresponding azide (III) was prepared from the hydrazide with the calculated amount of nitrous acid without affecting the urea part of the molecule. When the azide was boiled with absolute alcohol, the corresponding ethyl urethane (IV) resulted. The urethane was hydrolyzed with barium hydroxide to the triamine (V), thus opening the urea ring, and, at the same time, hydrolyzing the ethyl urethane group (*cf.* Fig. 2). The triamine (V) was then oxidized under the same conditions employed for the diaminocarboxylic acid, but no adipic acid could be identified among the oxidation products. This established the identity of one of the two carboxyl groups of the adipic acid with that originally present in the diaminocarboxylic acid, and it became evi-

dent that biotin contains the side chain  $-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{COOH}$  attached to one of its carbon atoms.

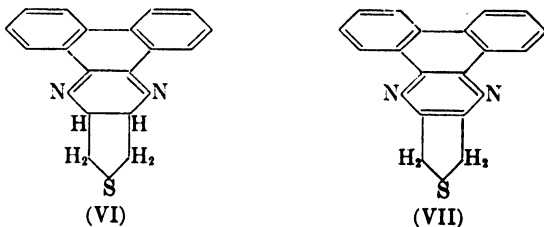
#### 4. The Nature of the Urea Ring

The formation of the diaminocarboxylic acid from biotin has been mentioned previously. It was shown that on treatment of the diaminocarboxylic acid with phosgene, biotin is regenerated, thus proving the presence in biotin of a cyclic urea structure. However, these experiments did not indicate whether a five- or a six-membered urea ring is present.

The size of the urea ring could be established in the following way. The diaminocarboxylic acid was condensed with phenanthraquinone, and a crystalline quinoxaline derivative,  $\text{C}_{23}\text{H}_{20}\text{O}_2\text{N}_2\text{S}$ , was thus obtained (39). The formation of quinoxaline derivatives by the condensation of 1,2-diamines with ortho-quinones is well established, and there is no evidence that 1,3-diamines form ring structures by this reaction. Two different compounds, dihydroquinoxalines or quinoxalines, may be obtained from the condensation of nonaromatic 1,2-diamines with phenanthraquinone as illustrated below.



It was suspected from the analytical composition, as well as from certain color reactions, that the condensation product derived from biotin is a quinoxaline rather than a dihydroquinoxaline derivative. A spectroscopic study enabled a definite decision in favor of the former possibility. The dihydroquinoxaline (VI) as well as the quinoxaline (VII) were prepared by condensation of 3,4-diaminothiophane (40) with phenanthraquinone.



The ultraviolet absorption curves of these derivatives were then compared with the absorption curve of the quinoxaline derived from biotin.

As shown in Fig. 3 the absorption curve of the derivative obtained from biotin was almost identical with that of the quinoxaline derivative from 3,4-diaminothiophane, and bore little resemblance to the curve of the dihydroquinoxaline derivative. This was a strong indication that the derivative formed from phenanthraquinone and the diamino carboxylic acid is a dibenzoquinoxaline derivative.

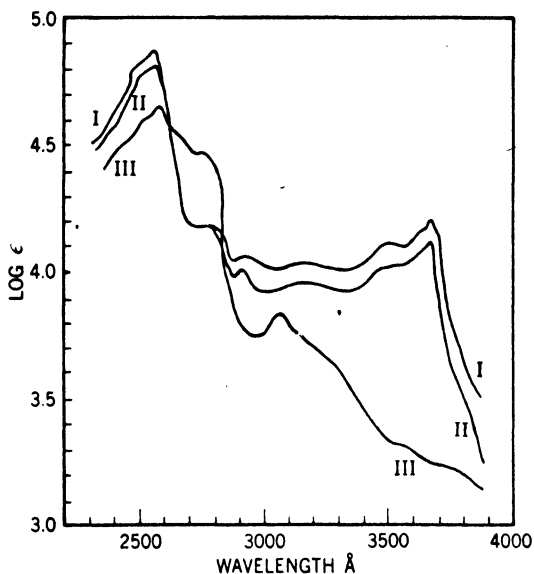
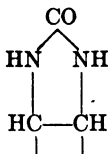


Fig. 3.—Ultraviolet absorption spectra of condensation product of phenanthraquinone with (I) the diaminocarboxylic acid from biotin; (II) 3,4-diaminothiophane, quinoxaline; (III) 3,4-diaminothiophane, dihydroquinoxaline.

The fact that the diaminocarboxylic acid forms a well characterized quinoxaline derivative when condensed with phenanthraquinone, established the 1,2-diamine structure for the diaminocarboxylic acid, and provided definite proof of the presence of a five-membered urea ring in biotin. The observation that this derivative is a dibenzoquinoxaline rather than a dibenzo-dihydroquinoxaline derivative, demonstrated further that biotin must contain the following structural element in which the carbon atoms bearing the amino groups carry hydrogen atoms.



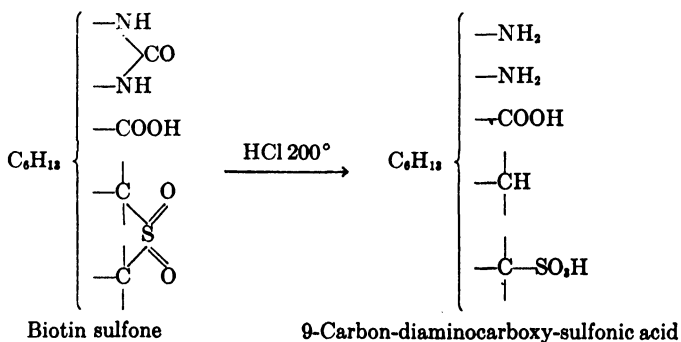


### 5. Kögl's Attempt to Demonstrate a Sulfur Ring in Biotin

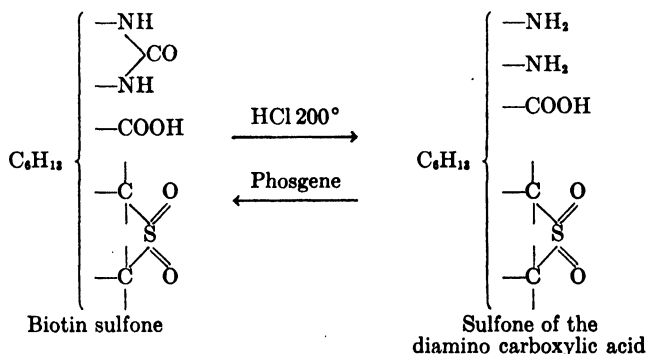
From the hydrogen content, the absence of an ethylenic linkage and the knowledge of the functional groups, it could be calculated that biotin must contain a bicyclic ring system. This fact, in conjunction with the evidence on the side chain and the urea ring, forced the conclusion that the sulfur atom must be part of a ring system.

Kögl and de Man (36) described experiments whereby they attempted to demonstrate the presence of a sulfur ring in biotin. Biotin sulfone was hydrolyzed at 200° with concentrated hydrochloric acid, and a new compound was obtained to which they assigned the structure of an aliphatic 9-carbon-diaminocarboxy-sulfonic acid. The following mechanism was suggested to explain the formation of this compound.

It was assumed that in addition to the opening of the urea ring, which results in the formation of the sulfone of the diaminocarboxylic acid, one of the carbon-sulfur linkages would be ruptured to yield the open chain sulfonic acid derivative as indicated below.



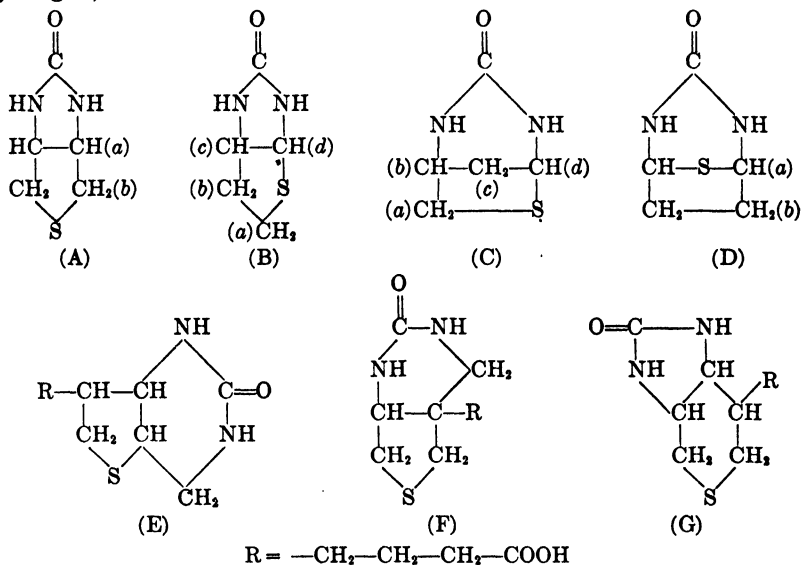
Kögl's experiments were repeated (41), and it was found that treatment of biotin sulfone with concentrated hydrochloric acid at 200° results only in the formation of the sulfone of the diaminocarboxylic acid, and did not alter the sulfur part of the molecule. This was demonstrated by an experiment in which biotin sulfone was resynthesized in 90% yield by the treatment of Kögl's hydrolysis product with phosgene.



Drastic hydrolysis of biotin sulfone does not affect the sulfur part of the molecule and, therefore, cannot be taken as evidence for the presence of a sulfur ring in biotin.

#### 6. The Structure of Biotin

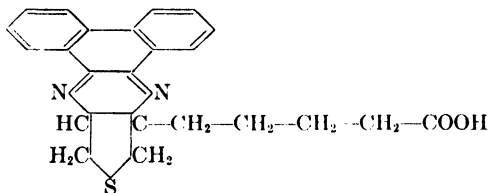
The following biotin structures were considered by du Vigneaud, Hofmann and Melville (42) on the basis of their initial degradation studies (*cf.* Fig. 4).



The side chain  $\text{—CH}_2\text{—CH}_2\text{—CH}_2\text{—CH}_2\text{—COOH}$  may replace the hydrogen atom at one of the positions indicated by *a*, *b*, *c* or *d*.

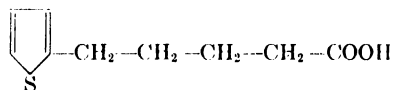
Fig. 4.

The great stability of the diaminocarboxylic acid toward hydrolytic agents rendered unlikely structures containing sulfur and nitrogen attached to the same carbon atom, and consequently structures (B), (C) and (D) were disregarded. The demonstration (39) that biotin contains a five-membered urea ring eliminated structures (E) and (F), leaving only structures (Aa), (Ab) and (G). The diaminocarboxylic acid derived from structure (Aa), when condensed with phenanthraquinone, could form a dihydroquinoxaline of the following structure:



but would not be expected to form a quinoxaline derivative. Since it had been shown (39) that the condensation product of the diaminocarboxylic acid with phenanthraquinone is a quinoxaline, structures (Ab) and (G) remained as the only possibilities. It may be noticed that structure (G) contains an *n*-butyric acid side chain attached to the ring system. Structure (G) might be a possibility if one assumes (42) that the adipic acid arises from the decarboxylation of a malonic or  $\beta$ -keto acid derivative, formed during the oxidation of the diaminocarboxylic acid.

Based on the following experiments (43) a decision between structures (Ab) and (G) was reached. The diaminocarboxylic acid was exhaustively methylated by means of dimethyl sulfate and alkali, and the methylation mixture was decomposed with strong hydrochloric acid. A sulfur-containing acid was thus obtained which was identified as  $\delta$ -(thienyl-2)-valeric acid.

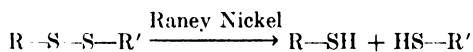


$\delta$ -(Thienyl-2)-valeric acid

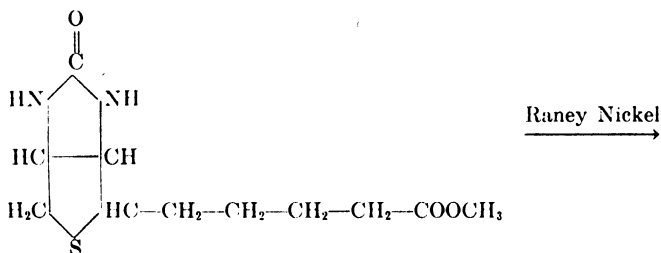
The formation of this degradation product from the diaminocarboxylic acid demonstrated the presence of a five-membered sulfur ring in biotin with an *n*-valeric acid side chain attached in position 2, and afforded conclusive evidence that formula (Ab) represents the molecular structure of biotin.

The same conclusions were reached by an independent approach. Bou-

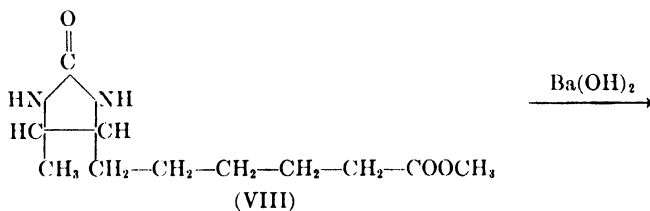
gault, *et al.* (44), have shown that treatment of disulfides with Raney Nickel in alcohol solution results in the following cleavage of the molecule:



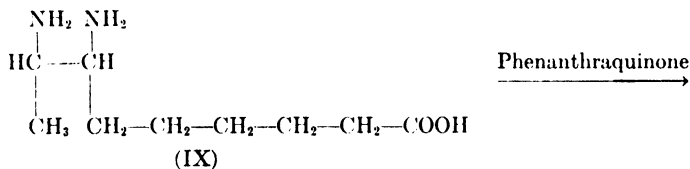
An analogous reaction effective with thioethers has been applied successfully to eliminate the sulfur from biotin (45). Accordingly biotin methyl ester was treated with Raney Nickel and desthiobiotin methyl ester (VIII) was obtained. Treatment of this compound with strong barium hydroxide brought about the formation of the diaminocarboxylic acid (IX) which, when condensed with phenanthraquinone, resulted in the formation of the quinoxaline (X).



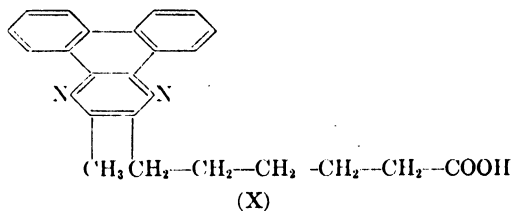
Biotin methyl ester



(VIII)



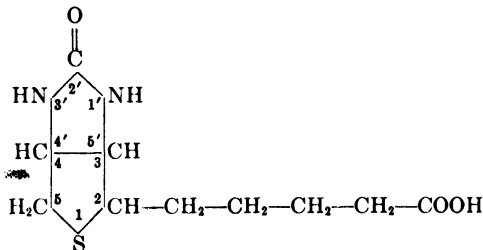
(IX)



(X)

The optical activity of the molecule was thus lost, and a comparison with synthetic compounds was facilitated.

The quinoxaline (X) was found to be identical with the quinoxaline prepared from synthetic 7,8-diaminopelargonic acid, confirming that biotin is 2'-keto-3,4-imidazolido-thiophane-(2)-*n*-valeric acid.



Biotin

## VI. Occurrence of Free and Bound Biotin in Nature

With the recognition of the fundamental importance of biotin in cell metabolism, it became necessary to obtain information as to the distribution of this factor in nature. It was soon realized that only a small fraction of the biotin present in tissues is extractable with water, and that most of it occurs in a bound form. Autolysis was found to liberate considerable amounts of biotin, and, consequently, comparative studies (46) of the biotin content of tissue autolysates were undertaken.

More careful and systematic investigations on the liberation of bound biotin demonstrated that autolysis liberates only a part of the amount present in tissues, and, in view of this fact, the previous results were found to be inadequate (47). The following table illustrates the amounts of biotin extracted from rat organs by different treatments.

TABLE III

LIBERATION OF BIOTIN FROM RAT TISSUES BY DIFFERENT TREATMENTS ACCORDING TO WILLIAMS

Amounts of biotin are expressed in  $\gamma$  per gm. of dry tissue

	Liver	Brain	Muscle
Hot water	0.056	0.0077	0.0098
Autolysis	0.53	0.0058	0.0036
Acid hydrolysis	2.30	0.27	0.11
Enzymatic digestion 48 hours	3.10	0.22	0.098

It may be seen from this table that acid hydrolysis and enzymatic digestion liberate maximum amounts of biotin from tissues. Biotin was found to be stable toward acids, but was rapidly destroyed when heated with 6 *N* sodium hydroxide. Hydrolysis with 4 to 6 *N* sulfuric or hydrochloric acid for two hours at 120° proved to be a satisfactory procedure for the quantitative liberation of biotin from natural materials. Lampen, Bahler and Peterson (29) made the observation, however, that treatment of crude materials with 4 *N* sulfuric acid frequently resulted in considerable loss of biotin. In view of this fact great care has to be exercised in the liberation of biotin from natural materials. Values on the biotin content of tissues should not be accepted as final before careful checks have been made, and different conditions have been used for its liberation. Values on the biotin content of various natural materials, using acid hydrolysis for the liberation of the biotin, have been reported by Lampen, Bahler and Peterson (29).

As mentioned previously, a small part of the biotin in tissues, usually referred to as "free" biotin, is extractable with cold water. It is questionable, however, whether the activity of such cold water extracts is due to free biotin or to physiologically active high molecular complexes of biotin with certain tissue constituents.

The presence of such physiologically active combinations has been demonstrated by György (48), who prepared saline extracts from egg yolks which he tested for biotin activity before and after dialysis. Dialysis did not alter the biotin content, and it was therefore concluded that the biotin was present in physiologically active combination with high molecular materials. Steaming of the extracts liberated the biotin from the combination and made it freely dialyzable.

Further investigations are needed on the biotin content of tissues as well as on the many different combinations in which this substance seems to exist in nature. Such studies should provide the basis for a better understanding of the mechanism of biotin action.

## VII. Avidin, the Substance in Egg White, Responsible for the Production of Egg White Injury\*

The cure of egg white injury with crystalline biotin, which established the identity of biotin with vitamin H, influenced decisively all of the recent developments in this field, since it made possible the replacement of the very time-consuming and inaccurate animal assays by the simple, accurate

\* The name antibiotin factor has likewise been proposed for this substance (49).

and fast yeast growth method. Eakin, McKinley and Williams (50) compared the biotin content of the tissues of normal chicks with the tissues of chicks fed a raw egg white diet. They found that the birds reared on the egg white diet were deficient in biotin despite the fact that an abundant supply of the vitamin was available with the diet. These findings indicated that the dietary biotin was not available to the animal when it was given in combination with egg white. In a later study (51) the *in vitro* effect of egg white on biotin was demonstrated by the fact that yeast was unable to grow in a biotin-containing culture medium to which unheated egg white had been added. Steaming of the egg-white-containing medium prior to inoculation with the yeast cells destroyed the toxic effects of the egg white, and normal growth was observed. Biotin thus combines with one of the constituents of the egg white to form a stable complex from which it cannot be recovered by dialysis. The combination between the active material and biotin is stoichiometric, and yeast is unable to utilize biotin in the combined form. Eakin, Snell and Williams (52) isolated the active material from egg white using the following procedure:

Egg white was coagulated with acetone, and the coagulate was washed with water. The washed cake of coagulated material which contained the active substance was extracted with 2% ammonium sulfate solution and the extracts were fractionated with ammonium sulfate, the active principle being precipitated by complete saturation with ammonium sulfate. The precipitate was dialyzed free of salts, was redissolved and precipitated with acetone.

The material prepared in this way gives the usual protein tests, and was assigned the name avidin.\* Assuming a ratio of 1 mol of biotin to 1 mol of avidin in the avidin biotin complex, a tentative molecular weight of 43,500 has been calculated for avidin.

Woolley and Longsworth (49) confirmed the above results by showing that the growth of *Clostridium butylicum* was inhibited by the addition of egg white to the biotin-containing culture medium. They isolated avidin by the use of essentially the same procedures as employed by Eakin, Snell and Williams and calculated a molecular weight of  $\leq 70,000$  for the substance. Their product, studied in the Tiselius electrophoresis apparatus, as well as in the ultracentrifuge, was found to behave as a single component. The isoelectric point of the preparation was found to be at pH 10 and this rather alkaline isoelectric point suggested to the authors that, in general, basic proteins might combine with biotin. Consequently the proteins salmin and nucleohistone from liver were tested for their combining power with biotin. The results were negative.

\* This name is derived from "avidalbumin," which literally means hungry albumin.

In a recent note (53) the preparation of crystalline avidin has been reported, the activity of which was found to be lower than that of the amorphous preparations.

That avidin is actually the agent responsible for the production of egg white injury, was demonstrated by György, *et al.* (54). These investigators were able to produce egg white injury in rats fed cooked egg white to which small amounts of the purified avidin had been added.

The biotin present in the diet as well as in the intestines combines with the avidin, and the avidin biotin complex passes unchanged through the alimentary tract and can be demonstrated in the feces (55).<sup>\*</sup> The biotin in the feces of rats fed a stock diet is present in a bound form from which it cannot be released by steaming, whereas the biotin in the excreta of animals fed avidin is liberated by this latter treatment. Since it had been previously shown that steaming splits the avidin biotin complex, these experiments were a demonstration of the presence of the avidin biotin complex in the feces.

Avidin is a protein which deserves special interest, not only because of its power to combine with biotin, but also because of its remarkable stability toward enzymatic digestion.

Very little information is available as to the type of linkage that is responsible for the formation of the avidin biotin complex, and only one study (56) has been published on the specificity of this unique reaction. A degradation product of biotin, namely, the diaminocarboxylic acid, has recently been tested for its combining power with avidin. This derivative was found to contain 10% of the physiological activity of biotin as measured by the yeast growth method, and this activity was not inhibited by the addition of avidin. It was, therefore, concluded that the urea ring in biotin is necessary for the combination.

In the light of these recent developments egg white injury must be regarded as an induced biotin deficiency, caused by avidin, a constituent of raw egg white, which has the ability to combine with the biotin and thus make it unavailable to the animal organism.

### VIII. The Role of Biotin in Animal Nutrition

Nutritional disorders caused by the feeding of egg white have been observed in several animal species; such as, the chick (7), the rabbit, the monkey (57) and the mouse (21). Observations have likewise been re-

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<sup>\*</sup> Parenteral administration of the avidin biotin complex cures rats from egg white injury (55).



ported by Sydenstricker, *et al.* (58, 59), on disorders in humans caused by diets rich in egg white. A group of volunteers, given a diet in which 30% of the total calories were supplied in the form of desiccated egg white, developed the following symptoms:

In the third and fourth weeks a fine scaly dermatitis was noticed, which subsequently spontaneously disappeared. In the fifth week one of the group developed a mild depression which progressed to an extreme lassitude and hallucination. Two others became slightly panicky. The only striking observation in the seventh and eighth weeks was a marked pallor of the skin. In the ninth and tenth weeks a form of dermatitis appeared which was similar to that observed during the beginning of the experiment. The biotin excretion, which on a normal diet was found to be 29–62  $\gamma$  per 24 hours, dropped to 3.5–7.5  $\gamma$ . Treatment of the patients with a biotin concentrate corresponding to a daily dose of 150–300  $\gamma$  brought relief in a few days.

It has not been possible as yet to produce a biotin deficiency in rats without the addition of egg white or avidin to the diet. Since a number of bacteria have the ability to synthesize biotin (60), bacterial synthesis in the intestines is the most likely explanation for the fact that the rat is more or less independent of a dietary supply of the vitamin. A true biotin deficiency has, however, been produced in the chick without the use of egg white. Ansbacher and Landy (26), using a heat-treated diet, produced a scaly dermatitis in chicks which was rapidly cured by the injection of biotin methyl ester or crude biotin concentrates.

A similar dermatitis had previously been reported by Hegsted, *et al.* (61), in chicks fed purified rations, and it was later found (27) that biotin is able to cure the animals. The biotin requirements of the chick seem to be higher than those of the rat, which makes the chick more dependent on a dietary supply of this substance.

Biotin has recently become of importance in connection with fat synthesis and the production of fatty livers in the rat. McHenry and Gavin (62) found that rats which are kept on a low fat diet develop acutely fatty livers when an alcoholic extract of beef liver is administered. The production of these fatty livers was not affected by choline, but was completely prevented when lipocaic was administered. It was later observed (63) that a similar fatty infiltration of the liver characterized by a high content of cholesterol occurred in rats fed biotin concentrates or pure biotin in conjunction with thiamine, riboflavin, pantothenic acid, pyridoxine and choline. Lipocaic, inositol or egg white were effective in preventing this "biotin" type of fatty liver.

### IX. The Possible Relationship of Biotin to Cancer

Since biotin is one of the most potent growth factors known, it became of interest to know whether rapidly growing tissues differ in their content of this vitamin. The first investigation in this direction was reported by West and Woglom (64), who made a comparative study on the biotin content of various normal tissues as compared with the corresponding embryo materials. *Rhizobium trifolii* was used as the test organism, and biotin was liberated from the tissues by acid hydrolysis. As these studies have been greatly extended (21), they shall be discussed later. West and Woglom's paper brought about much speculation as to the possible role of biotin in the production of malignancy (65).

TABLE IV

EFFECT OF BIOTIN ON THE PRODUCTION OF HEPATIC TUMORS BY *N,N*-DIMETHYLAMINO-AZOBENZENE

	Biotin preparation used	Amount of biotin added daily	No. of rats surviving	No. of rats with hepatic tumor
Expts. I, II, III	Controls	.....	28	1
	Crude and crystalline biotin	0.3-4.0 $\gamma$	50	22
Expts. II, III	Controls	.....	19	0
	Crystalline biotin	2.0-4.0 $\gamma$	16	9

Experiments in which biotin showed a procancerogenic effect in butter-yellow tumor formation were reported by du Vigneaud, Spangler, Burk, Kensler, Sugiura and Rhoads (66). These authors studied the effect of biotin on the production of the primary carcinoma of the liver as induced by the feeding of 1,1-*N*-dimethylaminoazobenzene (butter-yellow). They found that the addition of pure biotin to otherwise highly protective diets (67) caused a higher incidence in tumor formation. It was concluded that biotin is able to "break through" the dietary protection, and can exert a procancerogenic effect. Their findings are summarized in Table IV.

In the extended studies by West and Woglom (21) on the abnormalities in the distribution of biotin in certain tumors and embryo tissues, carefully selected tissue samples were employed for biotin analysis. A comparison was made of the normal adult tissues with the corresponding material from embryos and tumors. Some of their findings are summarized in Table V.

As may be seen from the table, striking differences were observed be-

tween the biotin content of embryonic tissues and tumors on the one hand, and the corresponding normal material on the other. In most cases studied, the tumor, as well as the embryonic tissues, contained significantly less biotin than did the normal controls. In a few cases such as rabbit skin tissue, however, the tumors and the embryonic tissues were definitely higher in the growth factor.

In another set of experiments the authors studied the influence of the biotin level of the host animal on the growth of certain transplantable tumors.

TABLE V

COMPARATIVE VALUES ON THE BIOTIN CONTENT\* OF NORMAL EMBRYO AND TUMOR TISSUES ACCORDING TO WEST AND WOGLOM

	Liver			Lung			Skin			Connective tissue		
	Normal	Embryo	Tumor	Normal	Embryo	Tumor	Normal	Embryo	Tumor	Normal	Embryo	Tumor
Rat	4480	1280	2030	.....	.....	.....	..	...	.....	2460	1280	544
Mouse	3613	2909	1170	2285	1330	1177	..	...	.....	1280	312	556
Rabbit	.....	.....	.....	.....	.....	.....	89	531	360† 369‡	..... 134	..... 1330	..... 813

\* Biotin values are expressed  $\mu\gamma$  per gm. of dry tissue.

† Rabbit papillomas.

‡ Rabbit skin carcinomas.

TABLE VI

WEIGHTS OF TRANSPLANTED TUMORS IN NORMAL AND BIOTIN-DEFICIENT MICE ACCORDING TO WEST AND WOGLOM

	Controls, gm.	Deficient, gm.
Average weight of sarcomas 37 after 17 days	0.36	0.26
Average weight of sarcomas 180 after 10 days	1.06	0.99

Mice were fed an avidin-containing diet until they developed a severe biotin deficiency and the biotin levels of their tissues had dropped 80 to 90% below normal. When the mice had about two to three weeks more to live, fragments of the rapidly growing sarcomas 37 as well as 180 were introduced subcutaneously into the deficient animals and into normal controls, and the growth of the tumors was observed. All of the tumors grew well producing large, healthy growth, despite the fact that the animals during the time were in an extreme stage of biotin depletion. After ten to seventeen days, respectively, the tumors were removed, and their weights recorded as summarized in Table VI.

Since only small differences in weight were observed between the normal and the biotin-deficient tumors, as may be seen from Table VI, it has been concluded that these two sarcomas are not dependent on biotin for the maintenance of their normal activity.

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# RECENT PROGRESS IN TUMOR ENZYMOLOGY

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## I. Introduction

The metabolism of living tissues is carried on by a diverse and intricate mosaic of enzymic catalysis. Under normal conditions and over the greater part of the life of the host, each tissue maintains a steady and consistent enzymic pattern. Under abnormal circumstances, this pattern may be altered by injury to one or more of the component enzymes. Thus, under conditions of dietary depletion in vitamins (1, 2) or essential metals (3, 4), the activity of certain tissue enzymes of the host are impaired. The introduction of toxic agents into the host may also result in enzyme impairment, such as the effect of carbon monoxide inhalation on respiratory ac-





TABLE I (Continued)  
RELATIVE ACTIVITY OF ENZYMES OR CONCENTRATIONS OF COMPONENTS IN HEPATOMAS, FETAL AND REGENERATING LIVERS<sup>a</sup>

Enzyme or component	Rat hepatomas induced with—				Mouse hepatomas <sup>b</sup>						Fetal Liver			Regenerating rat liver
	<i>p</i> -Dimethylaminoazobenzene		Aminooazotoluene		Aminooazotoluene induced in—			Spontaneous			Carbon tetrachloride-induced			
	Trans-planted	Pri-mary	Trans-planted	Pri-mary	I mice	C mice	C3H mice	C3H mice	A mice	A mice	Rabbit	Rat	Cat	
Creatine	100 (32)	.....	100 (31)	.....	.....	.....	.....	.....	100 (32)	100 (32)	.....	.....	.....	.....
Creatinine	100 (32)	.....	100 (31)	.....	.....	.....	.....	.....	100 (32)	100 (32)	.....	.....	.....	.....
Phosphatide	.....	.....	57 (33)	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Fatty acids	.....	.....	35 (33)	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Free cholesterol	.....	143 (9)	127 (33)	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Esterified cholesterol	.....	310 (9)	150 (33)	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Acid-soluble phosphorus	.....	.....	110 (33)	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Acid-insoluble phosphorus	.....	.....	65 (33)	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Vitamin A	.....	.....	.....	0 (34)	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Biotin	.....	45 (35)	.....	.....	30 (35)	.....	.....	.....	.....	.....	.....	.....	.....	.....
Copper	80 (36)	.....	.....	.....	60 (36)	.....	.....	80 (36)	.....	.....	.....	.....	.....	.....
Total solids	59 (12)	.....	63 (37)	.....	78 (12)	.....	.....	78 (12)	83 (12)	83 (12)	.....	.....	.....	.....
Total nitrogen	.....	.....	116 (37)	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Total phosphorus	.....	.....	125 (37)	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Total sulfur	.....	.....	124 (37)	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Sodium	.....	.....	163 (38)	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Potassium	.....	.....	487 (38)	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Calcium	.....	.....	61 (38)	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Magnesium	.....	.....	191 (38)	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Iron	.....	.....	66 (38)	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Iodine	.....	.....	100 (38)	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Chlorine	.....	.....	177 (38)	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Sodium chloride	.....	.....	177 (38)	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Ash	.....	.....	135 (38)	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....

<sup>a</sup> Values for normal adult liver, taken as 100, irrespective of species or strain. Figures in the table refer to the relative activity of each enzyme or concentration of each component on this basis. References are in parentheses. <sup>b</sup> Transplanted hepatomas. <sup>c</sup> Coenzymes I and II. <sup>d</sup> Probably owing to 2 or more separate enzymes. <sup>e</sup> Histologic evidence.

tivity. When a tissue becomes neoplastic, a change in one or more of the enzymes may occur. For present purposes, it is relatively unimportant to know whether such a change, if it does occur, is the cause of the neoplasia, the consequence of the neoplasia, or the result of processes external to the affected tissue. All that we wish to establish at the present time is whether a change in enzymatic activity is the specific accompaniment of neoplasia and to what extent this change may occur.

The form in which this review will be presented lays emphasis entirely on comparisons, on the one hand, of neoplasms and their normal tissues of origin, and, on the other hand, of similar tissues in normal and in tumor-bearing animals. Each tissue considered will be characterized by the activity of several enzyme systems or by the concentration of several organic and inorganic components. The discussion will center chiefly on the activity of individual, relatively well-defined enzymes rather than on factors such as tissue respiration and glycolysis which involve complexes of concurrent enzymic activities. No attempt is made to give anything more than a comprehensive summary of only those enzyme data which appear to the reviewer to be relevant to the cancer problem. Obviously the selection of such data is arbitrary but it is necessary for clarity.

## II. Comparison of the Enzymatic Activity of Tumors with that of the Normal Tissues of Origin

The data collected in Table I as well as those in succeeding tables are essentially comparative, and in every case reported the comparison has been made by the same investigator or groups of investigators. References to the appropriate literature are given in parentheses. The comparisons have thus been made throughout under the same conditions. Under the headings of the columns in Table I entitled "Rat hepatomas" and "Mouse hepatomas" appear those tumors which either arose spontaneously or were induced by the indicated carcinogen. All of the mouse hepatomas were transplants.

### 1. *Hepatomas and Liver*

Few tissues lend themselves so admirably to the comparative studies which form the basis of this review as do the liver and the hepatomas. These tumors arise in liver cells of different species and in various animal strains, either spontaneously or by induction through the administration of chemical agents. The liver contains a wide assortment of highly active enzymes. The possibility therefore exists of studying alterations in a

\* number of biocatalytic systems in hepatomas of different origin and in various strains and species of animals. Further comparison with growing hepatic tissues is afforded by the study of fetal and of regenerating liver in which, in contrast with the case of the hepatomas, the growth of the tissue is controlled.

The hepatomas which will be considered comprise those which were induced by the inclusion of *p*-dimethylaminoazobenzene (butter-yellow) or 2-amino-5-azotoluene in the diet of rats, and those which arose spontaneously in mice or were induced by the injection of 2-amino-5-azotoluene or of carbon tetrachloride. The rat hepatomas were first developed in Japan; transplantation of the aminoazotoluene-induced tumors was accomplished by Iikubo (see Shear (5) for review). Transplantation of butter-yellow-induced hepatomas was accomplished by White (6) at the National Cancer Institute. The chemical studies on the mouse hepatomas were performed on tumors developed in the latter institution by Andervont and Edwards. A recent paper by Andervont, Grady and Edwards (7) summarizes the histological characteristics of many of these mouse hepatomas.

There has been gathered into this section all of the available data on the enzymes and certain other components of the hepatomas and of related normal hepatic tissues. Some of the data have been obtained on the primary tumors, others on transplants. These will be distinguished. Where comparison of the same enzyme in the primary tumor and in the transplant has been possible, it was often found that the activity in the primary hepatoma was intermediate in value between that of the normal liver and that of the transplant. This is probably due in part to the presence of normal liver cells in the primary hepatoma.

The data so assembled are derived from the results of many investigators who used quite different experimental methods. This raises the problem of how to present the data most effectively within the limits set by the purpose of this review. The details of the various determinations and the methods of representing the data obtained are available in the original articles to which reference for such information may be made. The data have been assembled and compared on the following basis: the value for the activity of each enzyme in normal adult liver, irrespective of species or strain, is arbitrarily set at 100. The corresponding values of the enzymes in hepatomas or fetal or regenerating liver are then given on a comparative basis and will be less than or greater than 100 depending upon whether the activity of the enzyme concerned is respectively less or greater than that in normal liver. The values given for the former tissues thus represent the fractional part of the activity of normal liver and are based on the data obtained from the literature.

Examination of the data in Table I reveals on the whole striking differences in the activity of regenerating, of fetal, and of neoplastic liver tissues. With two exceptions, xanthine dehydrogenase activity and the content of total solids, regenerating rat liver is very similar in all respects to normal

adult (resting) liver. The activity of amylase in fetal liver is of the same order as that of adult liver, but the activity or concentration of nearly every other component studied in the former is invariably less than that found in the latter tissue. In the hepatomas, the activity of the enzymes may be less than, equal to, or greater than that of the corresponding enzymes in normal adult liver. With a few exceptions, most of the enzymes are diminished in the hepatomas as compared with normal liver, and so resemble the comparison of fetal with adult liver. The tissue components which are apparently more concentrated in the liver than in other body tissues, *e. g.*, arginase, catalase, riboflavin, etc., will be considerably diminished when the liver becomes neoplastic. It is possible that many of the hepatic enzymes and other components are simply stored in the liver, and the capacity (or necessity) for storage is lost when the liver becomes neoplastic. On the whole, however, it might be said that, enzymatically, fetal liver resembles the hepatomas more than it does either adult or regenerating liver. Burk (39) has shown that the glycolytic power of regenerating rat liver is very similar to that of normal adult liver, but that of fetal liver, like that of the hepatomas, is considerably greater than that of adult liver. Fetal liver contains a large amount of hematopoietic elements and hence the results obtained with this tissue must be accepted with some reserve.

The comparison of such obviously separate tissues as adult, fetal and regenerating liver and hepatomas offers the metabolic picture of tissues at definitely separable states. The data obtained are distinguishable on the basis of the state of the tissue. It would be advantageous however, to have more data on the intermediate stages involved, such as the change of liver into the neoplasm, the course of regenerating liver, and the growth of fetal into adult tissue. A few studies of the latter two changes are available (10, 35, 40, 41) and at least one study of the former (28). A dynamic picture is needed of the changes involved and this is obviously the most immediate task.

After this brief consideration of the general types of hepatic tissue it is appropriate to turn to the comparison of the hepatomas in each of the two species, rats and mice, in which these tumors have been studied. Hepatomas in rats have been induced by the feeding of two different carcinogenic chemicals. Most of the biochemical data on the butter-yellow tumors have been reported by American workers; that on aminoazotoluene tumors by the Japanese. Unfortunately, only a few reports of studies on the same chemical system in both hepatomas are available. In mice, however, there are considerable data on many enzyme systems all of which were studied in transplanted hepatomas of different origin in several inbred strains of mice (8). With these studies in mice supplemented by the few in rats, it has been possible to observe what effect the mode of origin of the tumor and the animal strain in which it arises have upon the enzymatic properties of the tumors. References to the various mouse tumors have been given (8).

**Arginase and Urea.**—Arginase, the enzyme which catalyzes the hydrolysis of arginine to ornithine and urea, is diminished to approximately

the same extent in hepatomas in rats and in various strains of mice. The results on the butter-yellow-induced hepatomas in rats from two different laboratories are quite similar. Of considerable interest is the observation that the arginase activity of the rat hepatomas and of the hepatomas of diverse origin in several strains of mice are very nearly the same. The content of urea in the hepatomas and in fetal liver is twice as great as normal. The urea in these tissues is probably due to the action of the arginase on arginine either derived from tissue protein breakdown or brought to the tissue by the blood stream. The urea-synthesizing mechanism, described by Krebs for normal, adult rat liver (42), is apparently missing in the rat hepatoma (30). The arginase component of the mechanism, although diminished, is still present; it is the synthetic portion of this cycle, *e. g.*, that responsible for the synthesis of arginine from citrulline or ornithine and ammonia which is deficient. A specific function of the normal liver thus apparently vanishes when the liver becomes neoplastic.

When ammonia is added to slices of liver or of hepatomas suspended in a glucose-containing medium, the rate of disappearance of the ammonia is distinctly greater in the former than in the latter tissue (30). The ammonia thus consumed is presumed to enter into a synthetic reaction with the glucose or its conversion products. In view of the rapid growth characteristic of the hepatoma it is somewhat surprising to find that the ammonia consumption is less than that of the normal liver, but it is possible that the synthetic processes of the tumor require other factors and perhaps other conditions than those employed.

**Catalase, Cytochrome Oxidase and Cytochrome c.**—These iron-containing components of normal liver are considerably reduced when the latter becomes neoplastic. The exact physiological function of catalase is not known; it is identified by its high rate of splitting of hydrogen peroxide. The lack of evidence for the presence of the latter substrate in animal tissues has been the cause of the inability to assign to catalase a definite and known function. Nevertheless, it appears to be an invariable component of the livers (and kidney and erythrocytes) of all mammals. Cytochrome oxidase is a participant in the respiration cycle in all tissues, and its substrate is the readily identifiable tissue component, cytochrome (43). Of the various cytochromes in tissues only the c component has been isolated. For the synthesis of catalase and of cytochrome oxidase by the tissues, a definite level of copper in the diet of the host animal has been found necessary (3, 4). In this connection it is interesting to note that the copper content of the hepatomas is lower than that of normal liver.

Catalase activity in the rat hepatoma is reduced to an almost negligible

amount as compared to that of normal liver. The reduction is not so marked in the mouse as in the rat hepatomas, but here too the activity is much less than normal and appears to be slightly related to the strain of mouse in which the hepatoma appears. Thus, the activity of catalase in the induced and in the spontaneous hepatoma in C3H mice is very nearly the same, but the catalase activity of the induced and spontaneous hepatomas in A mice is quite different in the two types of tumor and quite different also from that in the C3H mice. The cytochrome oxidase activity, however, is very nearly the same for hepatomas in all the mice strains.

The catalase (and arginase) activity of the induced tumor in *I* mice is the same as that found in the same tumor transplanted to  $F_1$  hybrids of this strain with C3H and with dilute brown mice (8), and thus hybridization, at least for the first generation, does not affect the enzymic properties of the tumor. Furthermore, in any one hepatoma in a given mouse strain the enzymic activity is invariably independent of the age or of the growth rate of the tumor.

Both cytochrome oxidase and cytochrome *c* are reduced to nearly the same extent in the rat hepatoma. This reduction is of some interest when it is considered that the respiration (in which these components participate) is practically the same in the hepatoma and in normal and regenerating liver (39). Shack has pointed out in an interesting calculation that the liver, whether resting or regenerating, has in contrast to the hepatoma, a considerable reserve of the oxidase-cytochrome system (11). Thus, when *p*-phenylenediamine in addition to glucose was added to liver and to hepatoma, the former tissue responded by a greater oxygen consumption than did the latter (18). Since the hepatomas do not possess an oxidative reserve, they can respond to demands for more energy by the supply of substrates only by an increase in glycolysis, whereas normal and regenerating liver (the latter, like hepatoma, also rapidly growing) may respond by increased oxidation (11). The oxygen consumption of the hepatoma is thus limited by the cytochrome system. This concept assists in the general interpretation of the high glycolysis of most tumors whose cytochrome oxidase values fall within the range of the hepatomas.

**Xanthine Dehydrogenase, *d*-Amino Acid Oxidase, Succinic Oxidase, Riboflavin, Thiamine, Coenzyme I, Vitamin A and Biotin.**—The enzymes considered in this section either have in common a known riboflavin structure or require riboflavin in the diet for their proper level of function (1, 2, 44). With one exception, xanthine dehydrogenase is present in greatly diminished activity in the hepatomas as compared with normal liver. The value for the hepatoma in the *I* strain is the same as that of normal liver.

The drop in the two hepatomas of the C3H strain is very nearly the same and the drop in the two hepatomas in the A strain is very nearly the same but different from that in the former strain. The riboflavin content of all the mouse hepatomas, however, is very nearly the same and is considerably lower than the value for normal liver. Since xanthine dehydrogenase, like *d*-amino acid oxidase and succinic oxidase, are flavin enzymes it is not surprising to find their activity reduced in the riboflavin-low hepatomas, but it is equally clear that there is no exact correlation between the drop in riboflavin content in the tumor, and the drop in either amino acid oxidase or xanthine dehydrogenase activity. Shack has suggested (11) that the lowering of the *d*-amino acid oxidase activity may be due to a deficiency in the protein component as well as in the flavin prosthetic group.

The oxidation chain in tissues is presumed to involve the action of molecular oxygen on cytochrome oxidase previously reduced by the reduced form of cytochrome. The latter in turn is then reduced by the flavoprotein enzymes. The iron and flavin systems are thus closely linked in the respiration cycle of animal tissues. Although both cytochrome oxidase and *d*-amino acid oxidase are reduced in activity when the liver becomes neoplastic, the latter is reduced to a much greater extent than the former. Furthermore, the fall in activity of the amino acid oxidase is much greater than that of xanthine dehydrogenase. Since the latter two enzymes are flavoproteins, and there appears to be more than enough flavin in the tumor to account for their combined activity, the unequal fall in their activity would lend weight to the suggestion that the fall is due to changes in the protein moiety of these enzymes. In any event, the change of liver cells into the neoplasm is thus accompanied by an unequal change in these as in many other enzyme systems.

Coenzyme I which participates in the oxidation mechanism contains nicotinic acid amide as a component and hence is also related to the vitamins. The content of coenzyme I and vitamin A in the hepatoma is considerably lower, that of thiamin only slightly lower, than that respectively in normal liver. The biotin values for the rat and mouse hepatomas are much lower than the corresponding values for the normal livers of these species. The lowered value in the case of the mouse hepatoma was characteristic only of the later growth of the tumor. A single specimen of a human liver carcinoma gave, in general, the same picture as that of the rat hepatoma.

**Amylase, Glycogen, Glucose and Lactic Acid.**—Amylase, which is probably composed of at least two separate enzymes (45) acts upon glycogen with the liberation of reducing groups. The activity of this enzyme



system in the hepatomas is very little different from that in normal liver. We have here, therefore, at least one system in liver which is not obviously affected by the change into a neoplasm. This is all the more interesting in view of the observation that the rat hepatoma is nearly devoid of glycogen. The mouse hepatomas, on the other hand, all contain glycogen in quantities which at present are not known. Thus, the amylase system is retained in the hepatoma independently of the capacity of the latter to store glycogen. Orr and Stickland (46) have pointed out that the substrate for glycolysis in the liver is glycogen, in the rat hepatoma it is glucose. Glycogen may thus be unnecessary in the hepatoma but if so the reason for the presence of the amylase system then becomes obscure. The relative proportion of glucose in the rat hepatoma is low, that of lactic acid is high, as compared to that of the normal liver, and this in view of the greater glycolysis in the former tissue is not surprising.

**Peptidases, Transaminase and Histidase.**—The proteolytic activity of the butter-yellow hepatoma in the rat is considerably greater than that of normal liver. The substrate used for these studies was hemoglobin. The proteolytic activity of the aminoazotoluene-induced rat hepatomas, when casein and gelatin were used as substrates, was found to be much less than that of normal liver. Proteolysis with the former tumor was accelerated by addition of cysteine, but with the latter tumor it was unaffected by addition of sulfhydryl. Although different conditions of extraction of the tissues were employed and different substrates were used, it is not believed that these were primarily responsible for the differences in the relative proteolytic activities shown. Reference to data on other enzymes in Table I, which were obtained with mouse tumors induced by different carcinogens in different strains, shows clearly that quite opposite results obtained even under the same conditions on the same enzyme in tumors in different strains are not exceptional, *e. g.*, thymonucleodepolymerase and alkaline phosphatase. The difference in proteolytic activity in the two hepatomas may well be ascribed to differences in the animal strains. The activity of dipeptidase and tripeptidase in the aminoazotoluene-induced rat hepatoma is the same as that of normal liver. It is indeed curious that the distribution in the enzyme sequence in the breakdown of the proteins should be so unequal when the liver becomes neoplastic. Like the case of the amylase system discussed above, the reason for the retention or loss of catalytic systems appears to be obscure.

Tumor growth involves the continuous synthesis of protein by the peptidases. This synthesis must be coupled with energy-producing mechanisms since the equilibrium between protein and peptides or amino acids

lies far on the side of hydrolysis. In view of the relatively low proportion of the oxygen-utilizing systems in the hepatomas and in fetal liver, the energy for synthesis in these tissues must be largely supplied by glycolysis. Burk has indeed shown that glycolysis is high in these tissues (39). The problem of relating biochemical synthesis to energy-producing mechanisms offers probably the most fundamental task in the elucidation of the neoplastic process.

Histidase, an enzyme so far found only in liver, which splits ammonia from the imidazole ring of histidine, is almost nonexistent in the rat hepatoma—another example of a specific hepatic function which practically disappears when the liver becomes neoplastic. Still another specific hepatic system which vanishes in this fashion is that responsible for the rapid degradation of cystine to  $H_2S$ ,  $NH_3$ , and pyruvic acid (66). Transaminase, which is concerned with the transfer of the amino group from certain amino acids to keto acids, decreases progressively in the liver during conversion of the latter into a hepatoma, finally reaching a low level characteristic of the hepatoma (28).

**Thymonucleodepolymerase and Phosphatase.**—The activity of the former enzyme, which is followed by the progressive depolymerization of sodium thymonucleate, is not very greatly changed in the hepatomas as compared to the normal liver. It is slightly below the normal in the rat and in one of the mouse hepatomas and slightly greater than normal in the rest of the mouse hepatomas. This system, therefore, like amylase and certain of the peptidases is practically completely retained when the liver becomes neoplastic.

In the case of acid phosphatase, the activity of this enzyme with one exception is significantly increased in both rat and mouse hepatomas. In the butter-yellow-induced rat hepatoma the augmentation of alkaline phosphatase activity is phenomenal, amounting to nearly 150 times that of the normal liver. It may be that this is due to the inclusion of bone elements in the tumor. The alkaline phosphatase activity of the mouse hepatomas varies from values lower than that of normal liver to values little different from that of normal liver. With this enzyme there appears to be a strain difference in mice, similar to the case of xanthine dehydrogenase and of catalase. Thus, the activities in hepatomas in the C3H strain are both lower than normal, those in hepatomas in the A strain are both nearly the same as normal. The lack of correlation in each of the tumors of the relative values for thymonucleodepolymerase and for phosphatase indicates that the two enzymes are quite different. The macro determinations of

phosphatase in the hepatomas have been independently confirmed by histologic determinations (47).

Since the thymonucleodepolymerase acts upon an exclusively nuclear substrate, this enzyme may be assumed to exist in the nucleus. It is interesting to note therefore that nuclear activity in adult, in fetal, in regenerating, and in neoplastic hepatic tissue, in so far as the state of nucleic acid is concerned, is practically the same. The activity of the factor responsible for the state of thymonucleic acid in the nucleus is thus independent of the rate of hepatic tissue growth. The proportion of desoxynucleoprotein in rat liver and in transplanted rat hepatomas, as well as the analytical composition and physical properties of the preparations from each of these tissues, is practically the same (26). It is possible that differences between liver and hepatoma must be sought for rather in the cytoplasmic components of these tissues.

**Phosphatide, Fatty Acids and Cholesterol.**—The content of phosphatides and of fatty acids in the rat hepatomas is lower than in normal liver but that of both free and esterified cholesterol is distinctly greater. These results are the same for hepatomas arising through butter-yellow or through aminoazotoluene administration. That a qualitative difference as well as a quantitative difference in the hepatoma cholesterol may exist is indicated by the report of the presence of 7-dehydro-cholesterol in the cholesterol fractions of the hepatoma (48).

**Ascorbic Acid and Glutathione.**—The analysis of tissues for these components is beset by many technical difficulties, and data reported must be accepted with some reserve. The ascorbic acid content and the glutathione content of the butter-yellow-induced hepatoma in rats have been reported to be lower, those in the aminoazotoluene-induced rat hepatomas to be distinctly higher than the corresponding values for these components in normal rat liver. On the other hand, the glutathione content of the mouse tumors investigated was about the same as that of normal mouse liver. The sulfhydryl groups of normal liver, available to titration after denaturation of the liver proteins, are greater in number per gram of tissue than the corresponding groups in the proteins of the hepatoma (26). This is interesting in view of the higher sulfur content of the hepatoma, but the difference may be explicable on the basis that more of the sulfur in the hepatoma may be in the form of disulfide, rather than sulfhydryl, groups.

Comparison of the ascorbic acid plus glutathione content of the two kinds of rat hepatoma reveals the greater reducing environment which must exist in the hepatomas which were induced by aminoazotoluene. It is possible that the failure to cause increased proteolysis in extracts of this

hepatoma by addition of reducing agents may have been due to the presence of already maximal quantities of these agents.

**Nitrogen and Creatine.**—The content of nonprotein N, of amino N, and of creatine and creatinine is practically the same in the hepatomas as in normal liver. Creatine is formed by the interaction of choline, methionine, glycine and arginine (49, 50). The amidination of glycine may occur in the kidney, but the methylation of the guanidoacetic acid produced definitely occurs in the liver (51). It is of considerable interest to note that the capacity to produce creatine (and creatinine) which represents a phase of amino acid metabolism, is yet another system which is apparently completely retained when the liver becomes neoplastic.

**Total Solids and Metals.**—The proportion of water in the hepatomas irrespective of origin, strain or species of animal appears to be distinctly higher than in normal liver. If this water is mainly extracellular it might be expected that the sodium and chloride ion concentrations would also be higher in the tumors. This is indeed the case. The proportion of potassium in the tumor is phenomenal. Iron is lower in the tumor than in normal liver and this is in keeping with the finding of decreased iron-containing enzyme systems in the hepatomas.

## 2. *Lymphomas and Lymph Nodes*

In Table II are gathered the data obtained on a transplanted lymphoma in A mice and the normal lymph nodes of this strain which are used as control tissues. A brief histologic description of this tumor has been given (10).

TABLE II  
LYMPHOMA AND NORMAL LYMPH NODES  
(Method of Representing Data Similar to that in Table I)

Enzyme	Lymphoma
Arginase	130 <sup>a</sup> (10)
Urea	20 <sup>a</sup> (10)
Catalase	50 <sup>a</sup> (52)
Xanthine dehydrogenase	960 <sup>a</sup> (52)
Thymonucleodepolymerase	1 <sup>a, b</sup> (53)
Acid phosphatase	20 <sup>a</sup> (54)
Alkaline phosphatase	125 <sup>a</sup> (54)

<sup>a</sup> Transplanted, subcutaneous lymphoma in A mice.

<sup>b</sup> Approximate.

Normal lymph nodes contain the greatest amount of thymonucleodepolymerase activity of all the animal tissues studied. This might be related to the relative size of the nucleus compared to the cytoplasm in this tissue. Since thymonucleic acid exists almost wholly in the nucleus, it might be expected that the enzyme or enzymes which affect this acid would be found in high activity in highly nuclear material. When the nodes become neoplastic, however, the thymonucleodepolymerase activity drops to a very low value despite the fact that the relative size of the nucleus remains fairly large in the tumor. The interrelation of morphology and physiological function is still obscure.

The activity of arginase and of alkaline phosphatase increases slightly in the lymphoma, that of xanthine dehydrogenase increases considerably, whereas that of acid phosphatase definitely decreases.

### 3. *Mammary Tumors and Breast*

The only data in the literature useful for comparative purposes are those obtained on spontaneous mammary tumors in mice. The control tissue has been either hyperplastic breast induced by subcutaneous injection of stilbestrol or lactating breast. The data are collected in Table III.

Hyperplastic breast is not an altogether unobjectionable comparison

TABLE III  
MAMMARY TUMORS AND BREAST  
(Method of Representing Data Similar to that in Table I)

Enzyme	Spontaneous mammary tumors
Arginase	170 <sup>a, c</sup> (10)
	173 <sup>b, c</sup> (10)
	248 <sup>a, d</sup> (10)
Catalase	100 <sup>a, c</sup> (52)
Xanthine dehydrogenase	150 <sup>a, c</sup> (52)
Thymonucleodepolymerase	100 <sup>a, c</sup> (53)
Acid phosphatase	116 <sup>a, c</sup> (54)
	111 <sup>b, c</sup> (54)
Alkaline phosphatase	244 <sup>a, c</sup> (54)
	222 <sup>b, c</sup> (54)

<sup>a</sup> In C3H mice.

<sup>b</sup> In A mice.

<sup>c</sup> Hyperplastic breast induced by stilbestrol as control tissue.

<sup>d</sup> Lactating breast as control tissue.

tissue for the spontaneous mammary tumors, and hence the comparisons made must be accepted with some reserve. Examination of the data in Table III reveals that the enzyme activity in the tumor is either equal to or distinctly greater than that in the hyperplastic breast. The activities of the various enzymes studied in tumors arising spontaneously in different strains are all the same, indicating the absence of any effect of the strain on the tumor enzyme. This apparent fact differentiates the spontaneous mammary tumors to some extent from the transplanted hepatomas in mice (Table I).

#### 4. *Rhabdomyosarcoma and Muscle*

There exists in the literature a single reference to work in which an enzyme system in a transplanted rhabdomyosarcoma and in normal muscle is studied (18). The data are given in Table IV.

The activity of the succinic oxidase is obviously greatly reduced in the rhabdomyosarcoma as compared with the normal muscle.

TABLE IV  
RHABDOMYOSARCOMA AND MUSCLE  
(Method of Representing Data Similar to that of Table I)

Enzyme	Rhabdomyosarcoma
Succinic oxidase	17 <sup>a</sup> (18)

<sup>a</sup> Transplanted tumor.

#### 5. *Adenocarcinoma of the Stomach and Intestine and Normal Gastric and Intestinal Mucosa*

Comparisons of the enzymatic activity of transplanted adenocarcinomas of the stomach and of the intestine in mice with the corresponding normal mucosa have been reported (55). The tumors were developed and described by Lorenz and Stewart (56, 57). The enzyme data are given in Table V.

Pepsin and rennin are enzymes specific to the gastric mucosa and apparently they vanish when this tissue becomes neoplastic. The greatest activity of alkaline phosphatase for all body tissues is found in the small intestine, and this enzyme also apparently nearly disappears in the intestinal adenocarcinoma. The gastric adenocarcinoma contains little or no alkaline phosphatase and in both types of adenocarcinoma the acid phos-

phatase is reduced to about half the activity of the normal mucosa. In contrast to these marked changes, the activity of thymonucleodepolymerase in the tumors is practically the same as in the respective normal tissues of origin.

TABLE V

ADENOCARCINOMA OF THE STOMACH AND INTESTINE AND NORMAL GASTRIC AND INTESTINAL MUCOSA

(Method of Representing Data Similar to that in Table I)

Enzyme	Gastric adenocarcinoma <sup>a</sup>	Intestinal adenocarcinoma <sup>b</sup>
Pepsin	0 (55)	...
Rennin	0 (55)	...
Thymonucleodepolymerase	98 (55)	98 (55)
Acid phosphatase	59 (54)	56 (54)
Alkaline phosphatase	0 (54)	0.1 (54)

<sup>a</sup> Transplanted tumor in C3H mice.

<sup>b</sup> Transplanted tumor in A mice.

There exists in the literature a report on the comparison of the tryptic, amylolytic and lipolytic activity of a single primary human adenocarcinoma of the pancreas with normal human pancreas (58). No difference was observed in the activity of these enzymes. This is an interesting finding but too great weight cannot be attached to observations on a single sample of tissue.

#### 6. *Carcinoma of the Prostate and Normal Prostate*

Kutscher and Wolbergs (59) first demonstrated the presence of a highly active acid phosphatase (optimum pH 5.0) in normal prostate tissue, but

TABLE VI

CARCINOMA OF PROSTATE AND NORMAL PROSTATE (HUMAN)

(Method of Representing Data Similar to that in Table I)

Enzyme	Tumor
Acid phosphatase <sup>a</sup>	156 (60)

<sup>a</sup> At pH 5.06.

the only comparison of this enzyme performed under the same conditions with carcinomatous prostate tissue is the report by Gutman and Gutman (60). Data by the latter investigators at pH 5.06 are given in Table VI.

The acid phosphatase in the carcinomatous prostate is definitely but not

greatly increased over that of the normal tissue. No data appear to exist in relation to the alkaline phosphatase activity of the tumor but Gutman, Sproul and Gutman (61) and Woodard (62) have reported that the normal prostate contains very little of this enzyme. Of considerable clinical importance has been the discovery that the serum of patients with disseminated carcinoma of the prostate contains large amounts of the acid phosphatase (60).

Additional information concerning the phosphatase activity of prostatic carcinoma has been derived from extensive studies on the sites of osteoplastic bone metastases secondary to carcinoma of the prostate gland (61). Studies of the lumbar vertebrae and ribs of a patient with disseminated prostatic cancer revealed high acid phosphatase values, suggesting that metastatic tumor cells arising from the tumor retain the high acid phosphatase activity of the primary cancer. It is necessary, however, to attempt to differentiate the activity of the phosphatase in the metastasis from that of the proliferating bone. Increased alkaline phosphatase activity is characteristic of growing bone in children and of hyperplastic bone (62) and of the osteoblastic type of osteogenic sarcoma (63). The bones which were studied in the case of disseminated prostatic carcinoma had not only a high acid but also a high alkaline phosphatase activity. Since the acid phosphatase of bones is very low in activity, the presence of this enzyme at the site of the metastasis betrays the presence of prostatic tissue, but the high alkaline phosphatase activity at this site must in turn be related in some fashion to the osteoplastic character of the prostatic metastasis. It seems indeed curious that determinations of the phosphatase activity at pH 9 of the primary carcinoma of the prostate have not been reported, for if this value were known it might be possible to separate the activity of the skeletal metastasis from that of the proliferating bone. It is interesting to consider in this connection the alkaline phosphatase values for the butter-yellow-induced hepatomas in rats (6, 54). The normal liver has a very low alkaline phosphatase activity but the hepatoma is extremely active in this enzyme. White and Edwards in a description of the pathology of this tumor (6) point out that the presence of this enzyme in considerable amount may be associated with the capacity for bone formation, and indeed in one of the transplants of this tumor membranous bone formation was observed. Although the studies on the skeletal metastases of prostatic carcinoma have been highly interesting and suggestive, it is unfortunate that only *skeletal* metastases have been investigated. The bone itself, and particularly growing bone, is rich in alkaline phosphatase and hence the results on this enzyme at sites of osteoplastic



metastases may be confusing. Further studies on the primary carcinoma or on metastases to nonosseous tissues low in alkaline phosphatase would go far in clearing up the problem.

### 7. Osteogenic Sarcoma and Bone

The chemical composition of bone has made extensive studies of the phosphatase mechanism in this tissue inevitable. The role of this enzyme in bone formation has been frequently reviewed (64, 65). Studies of the phosphatase activity in normal and neoplastic bone have been reported by several investigators (61, 62, 63, 66). Most of this work has been done with human tissues. Recently, an opportunity has arisen to study a transplantable osteogenic sarcoma in mice. The data are given in Table VII.

TABLE VII  
OSTEOGENIC SARCOMA AND BONE  
(Method of Representing Data Similar to that in Table I)

Enzyme	Tumor in mice <sup>a</sup>	Human Tumor
Acid phosphatase	270 (66)	250-1400 (67)
Alkaline phosphatase	262 (66)	1000 <sup>b</sup> (63)
		1000-17,400 <sup>c</sup> (63)
		170-128,000 (67)

<sup>a</sup> Transplanted tumors in C3H mice.

<sup>b</sup> Osteolytic type.

<sup>c</sup> Osteoblastic type.

Phosphatase (alkaline) has long been known to occur in large quantities at sites of ossification in embryos and in children (62, 65). It has been suggested that this enzyme hydrolyzed the phosphoric esters in the blood to bring about a local increase in the concentration of the phosphate ions, thereby leading to excess of the solubility product of the calcium phosphates which enter into the structure of bone (68). If phosphatase activity runs parallel with osteogenesis, it would be expected that the osteogenic tumors would be high in this enzyme, and the data given in Table VII reveal this to be indeed the case. It seems quite probable that phosphatase is synthesized by the osteoblasts.

The control tissues for the tumors described in Table VII were the long

bones of adult mice and of humans. If the growing bones of young mice or children had been selected for the control tissues, the ratios of tumor to control phosphatase would certainly be smaller. Nevertheless, the data reveal a marked increase in the bone phosphatase of the tumors, particularly of the osteoblastic type. The plasma phosphatase in patients with bone tumors also tends to increase markedly, and this will be considered in a separate section.

It is of interest to consider the marked similarity in the great increase of phosphatase activity in osteogenic sarcoma and in the bones at sites of osteoplastic metastases from primary carcinoma of the prostate. It has been suggested that certain metastases stimulate the production of alkaline phosphatase by osteogenic cells at the site of the metastasis (61). Whether the initiation of osteogenesis is a function of the metastatic tumor cell or whether the latter carries with it all the materials ready-made for osteogenesis including a large amount of alkaline phosphatase, can perhaps be better answered by study of the primary prostatic carcinoma or perhaps by metastases to nonosseous tissues naturally low in alkaline phosphatase. This question has been considered in the previous section.

### *8. General Considerations*

A survey of the data offered in the foregoing reveals that no simple generalization which covers the enzymatic behavior of all or even of any one group of tumors emerges at the present time. Such statements as that of Edlbacher (69), that all tumors are characterized by a higher arginase content than normal tissues, have been based on inadequate material and faulty comparison. Little advantage accrues to an over-simplification of a problem whose very complexity offers a fertile field of challenge.

When normal tissue becomes neoplastic certain enzyme systems may decrease in activity, others may increase, and still others may not change. The direction and order of magnitude of the change may depend on the species of animal, the strain in any given species, as well as the kind of tumor under consideration. When comparison is made of several tumors arising differently in different species and strains from the same kind of tissue such as the hepatomas, it is found that these hepatomas in mice may for certain enzyme systems be equal in activity, for other enzyme systems be equal in each strain but differ from strain to strain, and for other systems differ distinctly from tumor to tumor. Thus arginase and riboflavin belong to the first category; catalase, xanthine dehydrogenase, thymonucleodepolymerase, and to a certain extent acid and alkaline phosphatase,

to the second category; and amylase to the third category. These relations hold for mouse hepatomas. For mammary tumors in mice, not only arginase but also catalase and the phosphatases belong to the first category. For the lymphomas in mice, arginase belongs to the third category. Alkaline phosphatase is highly active in spontaneous mammary tumors in mice, but is present in negligible amounts in the breast carcinoma of humans (63). The normal prostate of man is rich in acid phosphatase but most species of animals contain this enzyme in very small amount in the prostate gland (70). Tumors in man which are strongly osteoplastic cause in general a distinct rise in the acid and alkaline phosphatase of the serum; in rats, however, which bear the butter-yellow-induced hepatoma containing phenomenal quantities of alkaline phosphatase, the phosphatase activity of the serum is entirely normal (54).

The behavior of the enzymes in a tumor in one species may not necessarily be the same as that of the enzymes in a tumor arising from the same kind of tissue in another species. Nevertheless, the picture given by all the enzymes in hepatomas, as an example, is more nearly the same in rats and in several strains of mice than it is like that given by all the enzymes in, say, mammary tumors or in lymphomas. It is necessary in the enzymatic characterization of tissues, whether neoplastic or otherwise, to have an enzyme "spectrum" or pattern in view. The study of one enzyme is insufficient. Many enzymes must be studied. Only in this way can the tissue be uniquely described, and the possible aberrations of individual enzyme systems be set against the proper background. A complete assay of such enzyme "spectra" in tumors arising from the same tissue in several different species is not yet available.

The range in activity for each of the enzymes so far observed among hepatomas, lymphomas, and mammary, gastric and intestinal tumors in mice taken all together is much narrower than that for each of the same enzymes in the normal tissues of origin of these tumors taken all together. In general, the range of enzymatic activity is much greater among normal tissues than among tumors. It is as if the tumors approached a common metabolic level (30, 66). The leveling effect is obviously due to the frequent loss in specific function when the normal tissue becomes neoplastic. This is most strikingly observed in the tissues above mentioned, when the neoplastic change involves the severe decrease in such highly active systems as hepatic arginase and catalase, lymphatic depolymerase, and intestinal alkaline phosphatase, etc. At least for mouse tumors, it would appear that enzymically the tumors are more nearly like each other than they are like normal tissues or than normal tissues are like each other.

### III. Comparison of the Activity of Enzymes and Concentration of Certain Components of the Tissues of Normal and of Tumor-Bearing Animals

#### 1. *Tissues Other than Blood or Serum*

The purpose of these studies is to observe whether any effect is elicited in the tissues of tumor-bearing animals at sites relatively far removed from the tumor. Whatever effect the tumor produces it can only do so in either of two ways, (a) by the liberation into the bloodstream of some material elaborated by the tumor, or (b) by the abstraction by the tumor of some component in the blood stream which is necessary for the maintenance or proper function of the distant tissue. Little or no evidence is available at the present time to decide between the alternatives for any one case; at the present time it is more important to collect the evidence for the presence of systemic effects elicited by tumors and to postpone possible explanations and interpretations for future consideration.

It is also important at the present time, however, to relate the systemic effect observed directly with the growth of the tumor so as to avoid the possibility that the effects produced may be the result of non-specified causes, such as cachexia or toxemia. For this purpose it is necessary to use animals in which the tumors are free of ulceration or infection, to observe whatever systemic effect is produced at intervals during the growth of the tumor and within the period when the animals appear to be healthy and vigorous, and finally to study several enzyme systems and other components in the various tissues under consideration. The last-mentioned recommendation is strongly urged because, whereas a general toxic condition may depress the activity of all or most of the enzyme systems of a tissue, a specific effect elicited by the tumor may be directed only toward certain select catalytic systems. Few studies in the literature meet all these criteria; many meet them in part, and the data selected, which represent the maximum systemic effect produced, are given in Table VIII. Like all the previous tables, the data are given on the basis of assigning a value of 100 to the tissue components of the normal animal and giving the values of the components of the same tissue in tumor-bearing animals relative to this figure.

Inspection of Table VIII reveals that in many tissues of the tumor-bearing animals profound changes from the normal values are apparent in certain enzyme systems and other components whereas no changes are apparent in other systems even in the same tissue.

**Liver.**—In rats bearing transplanted hepatomas or Jensen sarcomas or

TABLE VIII

COMPARISON OF THE ACTIVITY OF ENZYMES AND CONCENTRATION OF COMPONENTS OF THE TISSUES  
OTHER THAN BLOOD OR SERUM OF NORMAL AND OF TUMOR-BEARING ANIMALS<sup>a</sup>

Enzyme or component	Tissues of tumor-bearing animals				
	Liver	Kidney	Spleen	Muscle	Adrenals
Catalase	10 <sup>b</sup> (71)	80 <sup>c</sup> (72)	.....	.....	.....
	10 <sup>c</sup> (72)	50 <sup>d</sup> (72)	.....	.....	.....
	10-40 <sup>e</sup> (73)	20 <sup>f</sup> (72)	.....	.....	.....
	8 <sup>d</sup> (73)	.....	.....	.....	.....
	25 <sup>g</sup> (74)	.....	.....	.....	.....
Arginase	60 <sup>b</sup> (10)	28 <sup>g</sup> (77)	.....	>100 <sup>h</sup> (78)	.....
	61 <sup>i</sup> (75)	.....	.....	.....	.....
	100 <sup>j</sup> (10)	.....	.....	.....	.....
	100 <sup>c</sup> (76)	.....	.....	.....	.....
Xanthine dehydrogenase	100 <sup>b</sup> (41)	.....	.....	.....	.....
	100 <sup>j</sup> (52)	.....	.....	.....	.....
d-Amino acid oxidase <sup>o</sup>	95 <sup>b</sup> (11)	.....	.....	.....	.....
Riboflavin	60 <sup>b</sup> (15)	.....	.....	.....	.....
	72 <sup>b</sup> (12)	.....	.....	.....	.....
Cytochrome oxidase	96 <sup>b</sup> (11)	.....	.....	.....	.....
Vitamin A	97 <sup>l</sup> (79)	.....	.....	.....	.....
Cathepsin	12 <sup>m</sup> (80)	104 <sup>g</sup> (81)	171 <sup>g</sup> (81)	.....	.....
	148 <sup>c</sup> (76)	.....	.....	.....	.....
	143 <sup>m</sup> (76)	.....	.....	.....	.....
	131 <sup>g</sup> (81)	.....	.....	.....	.....
	100 <sup>i</sup> (82)	100 <sup>i</sup> (82)	100 <sup>i</sup> (82)	100 <sup>i</sup> (82)	.....
Glyoxalase	100 <sup>i</sup> (68)	.....	100 <sup>b</sup> (53)	.....	.....
Thymonucleodepolymerase	100 <sup>c</sup> (83)	.....	.....	50,000 <sup>c</sup> (83)	.....
Nuclease	.....	.....	.....	9,000 <sup>c</sup> (84)	.....
	.....	.....	.....	4,000 <sup>h</sup> (84)	.....
	.....	.....	.....	400 <sup>g</sup> (84)	.....
	.....	.....	.....	100 <sup>b,c,d</sup> (54)	.....
Acid phosphatase	100 <sup>b,c,d</sup> (54)	100 <sup>b,c,d</sup> (54)	100 <sup>b,c,d</sup> (54)	100 <sup>b,c,d</sup> (54)	.....
Alkaline phosphatase	100 <sup>b,c,d</sup> (54)	100 <sup>b,c,d</sup> (54)	100 <sup>b,c,d</sup> (54)	100 <sup>b,c,d</sup> (54)	.....
Esterase	36 <sup>c</sup> (85)	66 <sup>c</sup> (85)	.....	.....	.....
	85 <sup>m</sup> (86)	.....	.....	.....	.....
Lipase	36 <sup>c</sup> (87)	.....	.....	.....	.....
	91 <sup>n</sup> (86)	.....	.....	.....	.....
Phospholipid	50-72 <sup>o</sup> (88)	.....	.....	.....	.....
	85 <sup>b</sup> (89)	73 <sup>b</sup> (89)	87 <sup>b</sup> (89)	110 <sup>b</sup> (89)	75 <sup>b</sup> (89)
Free cholesterol	61 <sup>b</sup> (89)	143 <sup>b</sup> (89)	82 <sup>b</sup> (89)	87 <sup>b</sup> (89)	68 <sup>b</sup> (89)
Cholesterol esters	63 <sup>b</sup> (89)	161 <sup>b</sup> (89)	91 <sup>b</sup> (89)	41 <sup>b</sup> (89)	10 <sup>b</sup> (89)
Fatty acids	57 <sup>b</sup> (89)	42 <sup>b</sup> (89)	47 <sup>b</sup> (89)	59 <sup>b</sup> (89)	68 <sup>b</sup> (89)
Water content	101 <sup>b</sup> (89)	102 <sup>b</sup> (89)	101 <sup>b</sup> (89)	102 <sup>b</sup> (89)	104 <sup>b</sup> (89)
	105 <sup>b</sup> (12)	.....	.....	.....	.....
Copper	160 <sup>b</sup> (36)	84 <sup>f</sup> (36)	.....	.....	.....
	160 <sup>c</sup> (36)	55 <sup>c</sup> (36)	.....	.....	.....
	130 <sup>d</sup> (36)	.....	.....	.....	.....
	113 <sup>f</sup> (36)	.....	.....	.....	.....

<sup>a</sup> Value for normal tissue is taken as 100, and that of tissue from tumor-bearing animals is given on this basis. References are given in parentheses. <sup>b</sup> Rats bearing transplanted hepatoma. <sup>c</sup> Rats bearing transplanted Jensen sarcoma. <sup>d</sup> Mice of various strains bearing the rapidly growing transplanted sarcoma 37 or sarcoma 180. <sup>e</sup> Mice of various strains bearing transplanted pulmonary, gastric, brain, hepatic, melanotic and lymphatic tumors, and spontaneous mammary tumors. <sup>f</sup> C3H mice bearing spontaneous mammary tumors. <sup>g</sup> Mice bearing the Ehrlich carcinoma. <sup>h</sup> Rats bearing the transplanted Flexner-Jobling sarcoma. <sup>i</sup> Rats bearing transplanted Philadelphia sarcoma 1. <sup>j</sup> Mice of various strains bearing transplanted hepatic tumors. <sup>k</sup> Non-cancerous portion of liver with hepatoma induced by butter-yellow feeding. <sup>l</sup> Humans with gastro-intestinal tumors. <sup>m</sup> Horse with melanosis. <sup>n</sup> Rats bearing the Walker carcinosarcoma. <sup>o</sup> Mice bearing transplanted sarcomas, lymphomas or mammary carcinomas.

in mice bearing various transplanted tumors, the activity of the liver catalase is markedly depressed below normal, but the activity of xanthine dehydrogenase and of acid and alkaline phosphatase is equal to the normal value. The fact that the activity of xanthine dehydrogenase and *d*-amino acid oxidase is normal is of interest because of the lowered value for riboflavin concentration. Evidently there is still enough of the riboflavin present in the liver to account for the normal activity of these two enzymes. Certain other components appear to be augmented in the livers of tumor-bearing animals and these appear to be cathepsin and copper. The livers as well as other tissues of the tumor-bearing animals universally contain a somewhat higher water content than do the same tissues in normal animals.

Certain species differences are apparent, *i. e.*, arginase is decreased in the livers of tumor-bearing rats but not in the livers of tumor-bearing mice. Certain differences within the species are also apparent, for the hepatic esterase and lipase activity of rats bearing the Jensen sarcoma appear to be much more depressed in activity than in rats bearing the Walker tumor. The relative rate of tumor growth may explain this difference in part.

The enzyme system most thoroughly studied in the tissues of tumor-bearing animals has been catalase (72). Nearly invariably, the activity of this enzyme in the livers and kidneys of rats and of mice bearing a wide variety of tumors is lowered considerably below normal. It is of interest to point out that one of the earliest reports on the systemic effect of tumors was made by Brahn (90) who observed very low liver catalase values in human beings who had died as a result of various forms of cancer. The low liver catalase activity in various species of tumor-bearing animals is also frequently paralleled by low blood hemoglobin concentrations. Both catalase and hemoglobin are proteins which contain the same hematoporphyrin nucleus. There does not appear to be any direct effect of the tumor on the liver catalase molecule, for incubation *in vitro* of the extracts or slices of tumors with extracts or slices of liver or with solutions of crystalline catalase does not alter the activity of the catalase in any way (66). There is likewise no evidence for the presence of a dissociable inhibitor for catalase in the liver of tumor-bearing animals (66). It may be that the effect of the tumor on the liver catalase activity is due to an interference with the synthesis of the hematoporphyrin nucleus.

**Kidney, Spleen and Adrenals.**—Catalase, arginase, esterase and copper appear to be considerably reduced below the normal level in the kidneys of tumor-bearing animals. On the other hand, free and esterified cholesterol are markedly increased. The extent of the drop in the kidney catalase activity is not so great as in the liver and thus in most tumor-bearing

animals the kidney catalase activity is much greater, per gram of tissue than the liver catalase—a unique condition.

The catheptic activity of the spleen, like that of the liver, appears to be definitely increased in tumor-bearing animals. This tissue frequently hypertrophies in tumor-bearing animals. Other enzymes are little affected.

The content of various fats is lowered considerably below the normal in the adrenal glands of tumor-bearing rats.

**Muscle.**—A remarkable finding is reported concerning the practical induction of nuclease activity in the striated muscle of tumor-bearing rats and mice (83, 84). This enzyme, the activity of which is followed by the splitting of phosphoric acid from thymonucleic or ribonucleic acid, is either absent or present in negligible amounts in the muscles of normal animals. The extent of its appearance in the muscles of the tumor-bearing animals is unusual. The original observations of Edlbacher and Kutscher (83) were confirmed and extended by Wienbeck (84) in another laboratory. Further extensions of this observation are clearly desirable.

A definite increase in the arginase activity of the muscles of tumor-bearing animals has also been reported (78). The data in the original paper are, however, too sketchy to discuss in detail.

**Relation of the Growth Rate of the Tumor to the Systemic Effect.**—If the tumor is directly or indirectly responsible for producing an effect on a distant tissue of the tumor-bearing animal it might be expected that this effect would increase in intensity with progressive growth of the tumor. A few of the investigations into the study of the systemic effect have considered this possibility. Edlbacher and Kutscher (83) pointed out that the degree of induction of nuclease activity in the muscle tissue of tumor-bearing animals was proportional to the growth rate of the tumor. Fujiwara (77), Green (85) and others (72, 91) made similar observations for other enzyme systems in other tissues in tumor-bearing animals. Greenstein and Andervont (73) observed that sarcoma 37 cutaneously inoculated into Y, C and dilute brown mice grew rapidly and that the liver catalase activity of these animals dropped rapidly during growth of the tumor and at about the same rate in each strain. When the same tumor was inoculated subcutaneously into the tails of mice of these strains, it grew in all strains at about half the rate that it did when cutaneously implanted, and the rate of fall of the liver catalase activity was roughly half that observed in animals with tumors implanted in the latter manner. It has been emphasized recently (72) that the degree of the systemic effect observed has no meaning unless the corresponding weight and age of the tumor is known. In any event, all observations indicate that the systemic

effects elicited by the tumor run generally parallel in degree with the growth of the tumor. This is an important fact. For one thing, the available evidence indicates that the systemic effect changes in degree but not in kind during growth of the tumor, and hence the property of the tumor which is responsible for the systemic effect changes quantitatively but not qualitatively with growth of the tumor. The tumor at least as far as this property is concerned remains constant during its existence.

When a tumor grows extremely slowly it is often unlikely that a systemic effect will be evident. This has been observed in the case of certain slow-growing mouse tumors (73). It is possible that in the tumor-bearing animals a competition exists between the rate of the systemic effect produced in a tissue by the presence of a distant tumor and the rate of repair by that tissue. In the case of animals with rapidly growing tumors the former rate predominates and, conversely, the latter rate might predominate in the case of animals with the very slow-growing tumors.

Finally, it is possible that even in animals bearing rapidly growing tumors some systemic effect may be absent. This has been observed in the case of C57 black mice bearing sarcoma 37 (73). In this strain the enzymatic activity of the livers of the normal animals is much below that of the livers of many other mouse strains and this may be an explanation for the anomalous behavior. The enzymatic activity of the tissues of different strains of normal mice may vary considerably, and it is evident that this factor must be taken into account. The normal base level of activity of the tissue enzymes of available strains must be known and used as a frame of reference for any work in this topic.

**Reversibility of the Systemic Effect.**—If the presence of the tumor is directly responsible for the systemic effect produced in distant tissues of the tumor-bearing animal, it might be expected that complete removal of the tumor from the latter would result in the disappearance of the systemic effect and in the restoration of the distant tissue to its normal condition. This expectation has been fulfilled (71, 73). For permanent restoration the absence of metastases is taken for granted.

The catalase activity of the livers of rats bearing the transplanted hepatoma 31 is very much lower than that of the livers of normal rats (71). This tumor is encapsulated and readily removed from the animal when the latter is under ether anaesthesia. Within 48 hours following such an operation the liver of the operated animal has a normal catalase value. If a new tumor is implanted into the same animal the liver catalase activity again decreases, and again is restored to normal by a second operative removal of the tumor.



The catalase activity of the livers of C, Y<sub>1</sub> and dilute brown mice all bearing the intracutaneously transplanted sarcoma 37 is very much lower than that of the livers of the normal animals (73). Under those conditions of growth the tumor cannot be completely removed from the animal for it readily invades the surrounding tissues. Incomplete removal of the tumor from these animals causes a temporary rise in the liver catalase activity which is roughly proportional to the amount of tumor removed, and which quickly drops again as the tumor continues to grow. In order to remove the tumor completely from the mice, recourse was made to a technique developed by Andervont for immunity studies (92). Sarcoma 37 may be implanted in the tails of mice where it grows at a somewhat slower rate than it does in the abdominal skin. The drop in the liver catalase activity of the animals with the growth of such caudal implants is also proportionately slower. When the tails are amputated the tumors are completely removed from the animal. Within three days of this operation the liver catalase activity of the mice is restored to the normal level which is maintained thereafter (73).

Physical removal of the tumor is not the only evidence for the reversibility of the systemic effect. The transplanted sarcoma 37 grows progressively in I strain mice for about two to three weeks after implantation when it starts to regress spontaneously and after about five to six weeks it disappears completely. The liver catalase activity of I strain mice bearing this tumor drops with growth of the tumor and then rises with regression until it reaches the normal level again with disappearance of the tumor (73). This case provides a cogent illustration of the necessity of studying systemic effects in animals bearing progressively growing tumors.

The reversibility of systemic effects elicited in human serum by the presence of tumors has often been demonstrated following removal of the tumors. These cases will be reviewed below in the section on serum.

## 2. *Blood and Serum*

That marked and distinct changes in the character of the blood plasma as a result of cancerous growth might serve as a practical diagnostic test was early recognized by clinical workers in the field. Particularly in the case of suspected tumors in difficultly accessible sites, the blood might serve as a window through which the progressive development of the disease or its treatment could be made visible.

Recent types of serochemical diagnosis appear to be the result of an over-ready acceptance of the Kögl hypothesis concerning the presence of the unnatural *d*-amino acid optical isomers in tumors. The assumption was that if these isomers existed in peptide combination in the tumor there must be peptidases present in the tumor capable of catalyzing the hydrolysis of such peptides; during development of the tumor some of

these enzymes would presumably "leak" into the blood stream. The results collected have been highly indecisive; the data for the most part have been summarized by Maver, Johnson and Thompson (93) who questioned the specificity of the method. It has long been known that many normal tissues are capable of splitting peptides involving the unnatural amino acid isomers (94). Furthermore, one may perhaps doubt the evidence which rests upon the practice of conducting digestions at an elevated temperature of crude serum for the length of time (2-5 days) apparently needed in order to observe such reactions that have been reported. Finally, with the discrediting of the Kögl hypothesis any basis for the postulated serum reaction vanished.

To the reader who has studied the vast literature of cancer serochemistry, the subject has appeared to be more a sequence of psychological than chemical phenomena. The urgency of the immediate task has perhaps been the frequent cause of confusion. Perhaps the advisable approach would be not to insist at the very start that a rigorous, practical and specific test for malignancy be established, but that the readily observed changes in the blood or sera of tumor-bearing individuals be more closely examined, even if such changes appear to occur in conditions other than cancer. On further closer scrutiny such changes in the cancerous individual might be distinguished from those occurring in the noncancerous individual. Even in those cases in which this may turn out to be unsuccessful such information has an independent interest of its own. There has, therefore, been selected for discussion in this section that evidence which appears to the reviewer to be sufficiently authentic to illustrate systemic effects elicited in the blood of the tumor-bearing host. These are (a) a progressive depression in the blood hemoglobin level of many species of animals during the growth of many kinds of tumors, (b) a marked decrease in the serum esterase and lipase activity as a result of tumor growth in different species, and (c) the marked rise in serum phosphatase activity, particularly acid phosphatase, in prostatic carcinoma in man. Only the latter appears to be specific for cancer, but all three are closely associated with the growth of tumors.

**Blood Hemoglobin.**—Many investigators have pointed out that anemia generally accompanies cancer. The pertinent literature has been summarized by Taylor and Pollack (95). The anemic condition appears to be brought about in many species of animals bearing many types of malignant growths. Reliable evidence that there is a direct relation between the hemoglobin level and the course of growth of implanted as well as induced tumors in animals has been reported by Taylor and Pollack (95). The hemoglobin level decreased progressively to very low levels during the period of growth of the implanted tumor, during the precancerous stage in animals treated with carcinogens and during the growth of the induced

tumor. Strong (96) showed that the hemoglobin level of mice of high spontaneous tumor incidence falls off markedly even before the tumorous growth appears, in contrast to strains of low incidence in which this effect does not appear. It is suggestive, as mentioned previously in this review, that one of the most marked systemic effects produced in the tissue of the tumor-bearing animals, and one, which like the hemoglobin lowering, appears to occur in all species studied, is the lowering of catalase activity. Hemoglobin and catalase contain the same prosthetic group, the hema-toporphyrin nucleus, and it may be that the presence of the tumor interferes in some manner with the synthesis of this nucleus. Such possible interference by the tumor is, however, not equally distributed in all tissues; the liver catalase activity of tumor-bearing animals is depressed in extent much more than is the kidney catalase—the blood catalase is very little affected (72). It illustrates the relative independence of catalase and of hemoglobin within the erythrocyte and suggests the possibility that these two components enter the red cell from different sites.

**Blood and Serum Esterase and Lipase.**—Several independent investigators have clearly demonstrated that the activity of blood and serum lipase and esterase in tumor-bearing animals progressively decreases to quite low levels during growth of the tumor (85–87, 97–100). In rats with implanted tumors the activity may be reduced from a half to one-quarter of the normal activity, and the change begins at an early stage of the tumor growth. In animals in which the tumor begins to regress spontaneously the serum lipase activity rises and when the tumor has disappeared the enzyme activity reaches the normal level (85). The parallel behavior of the serum lipase and tumor growth or regression is good proof that the extent of the enzyme activity is closely associated with the cancerous state of the host animal. It may be pointed out that in the tumor-bearing animals the esterase activity of other tissues such as the liver and kidney is considerably lower than normal (Table VIII).

**Serum Phosphatase.**—Kay pointed out in 1930 (101) that the plasma phosphatase (at pH 7.6) was considerably elevated above normal in generalized diseases of bone. The rise in plasma phosphatase activity appears to be a reflection of the tendency to bone formation and thus occurs normally in growing children as well as in such diseases as rickets, hyperparathyroidism and osteogenic sarcoma whose only relation with each other consists in the fact that the host is attempting to repair or is growing bone. In the case of carcinoma of various tissues which involve osteoplastic metastases the alkaline phosphatase activity in the blood is also considerably elevated (61, 63, 102). Woodard and Craver (103) have

pointed out that the activity of the serum phosphatase is frequently high in lymphoid diseases long before bone changes become evident, and they suggest that the latter may occur more frequently in this group of diseases than formerly suspected. It seems clear at the present time that an elevation in the serum alkaline phosphatase activity, *in the absence of jaundice*, is evidence for the presence of osteoblastic activity in the host brought about by a variety of conditions. It remains true, however, that the highest values reported as a result of any one of these conditions occurs in cases of osteogenic sarcoma (63, 104). Removal of the osteogenic sarcoma by operation is followed by a drop of the plasma phosphatase to the normal level and recurrence of the tumor is again followed by a second rise (63). The effect of the tumor is thus a reversible one and similar to the experimental studies on the hepatomas in the section above on "Reversibility of the Systemic Effect."

Phosphatase activity in all tissues studied possesses two maxima at different pH levels, one at about a pH of 9 and the other at about a pH of 5. Perlmann and Ferry (105) have recently adduced evidence that the activity demonstrated at each of these levels is due to a separate enzyme. The normal prostate gland in man is rich in the acid phosphatase (59, 60). In primary carcinoma of the prostate with osteoplastic skeletal metastases the Gutmans (60) found not only an elevated alkaline but also an elevated acid serum phosphatase. This was an important observation for it enabled the clinician to differentiate the general osteoplastic character of the high serum alkaline phosphatase from the high acid serum phosphatase which appears to be specific for prostatic carcinoma (61, 106). The observation of the Gutmans has been amply confirmed by many subsequent investigators (107-112, 62). Indeed the level of acid serum phosphatase has with some confidence been used as a prognostic sign during the course of the disease. In general the higher this value the more unfavorable the prognosis has been found to be.

In prostatic cancer with marked elevation of the serum acid phosphatase, Huggins and Hodges (109) found that castration or injection of large amounts of estrogen produced in general a sharp reduction of this enzyme to or toward the normal range. Cession of administration of stilbestrol was followed by a rise in the enzyme activity and subsequent administration was again followed by a return to the normal level. In most cases castration or administration of stilbestrol was followed by clinical improvement (111). The serum acid phosphatase level is thus associated with the growth of the tumor and thus agrees with the other systemic effects described, such as the changes in liver catalase, blood hemoglobin and serum

lipase. In the case of the prostatic carcinoma, however, the relation between the tumor and the enzyme may be mediated by hormone control. The activity of the prostatic tissue as reflected by the rise of the acid serum phosphatase apparently depends in part upon the degree of androgenic hormone activity. This enzyme will thus vary according to the androgen control of the prostatic epithelium. The changes in the alkaline serum phosphatase activity bear little relation to the changes in the acid phosphatase since the former is apparently due only to the level of osteoblastic cells. Such changes as occur in the alkaline serum phosphatase will be of a secondary nature only. If the carcinomatous prostatic epithelium is inhibited by estrogens it might be expected that it would be activated by androgen injections, and this is indeed the case, *e. g.*, rise in the serum acid phosphatase above the pre-injection level was observed (109). Bilateral orchidectomy in dogs resulted in no change in the level of the serum phosphatases.

Attempts to influence the acid phosphatase activity of the prostate gland of both mature and immature rats by injection of various sex hormones were unsuccessful (70). However, further studies in experimental animals of the hormonal control of tumors of the sexual and accessory tissues await the production of such tumors in animals of appropriate strains. Such techniques as described by Shimkin, Grady and Andervont (113) offer much promise in this direction.

### 3. General Considerations

The marked changes from the normal values of the tissue components in tumor-bearing animals have been described as systemic effects elicited by the presence of the tumor. The fundamental criterion on which these effects rest may be expressed as follows: the effect must be progressive with the growth of the tumor, increasing as the tumor grows and decreasing if the tumor regresses or is operatively removed. According to this criterion the effect is *directly* related to the existence of the tumor, but so far we have little evidence as to whether such an effect is *specifically* related to the tumor. Nevertheless, the fact that pronounced effects occur in tissues relatively far removed from the site of the tumor lends weight to the contemporary viewpoint that cancer is not a local but a generalized disease.

Many other pathological states of the animal are recognized by systemic effects which rise in intensity with the progress of the disease and subside with its fall, *e. g.*, diabetes, certain psychotic states, thyroidism, etc. In this respect cancer is not a unique disease. But just as the effects elicited

by diabetes may be distinguished from those observed in pituitary disease, so it might be possible to relate specifically certain systemic effects in the tumor-bearing animal to the growth of the tumor. Whatever success has attended the former observations has been due largely to the ability to link the effects noted with the physiology of the affected tissue. We know very little of the physiology of tumors in general. The decrease in activity of catalase and esterase and the increase of phosphatase and nuclease in the tissue of tumor-bearing animals may or may not be related to each other. If more were known about the metabolism of the tumor the bearing of these phenomena on the growth of the tumor might be explicable. The study of systemic enzymatic effects elicited in the tumor-bearing animal by the presence of the tumor constitutes an indirect approach to the cancer problem in contrast with the direct approach offered by the study of the tumor itself. Each approach supplements the other and both are necessary for the elucidation of this complex problem.

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# THE ROLE OF MICROORGANISMS AND ENZYMES IN WINE MAKING

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Because fermentation, the process by which grape juice becomes wine, is caused by yeast and since the most important forms of spoilage of wine

are induced by bacteria, it is evident that microorganisms are of vital importance in wine making. The clearing of wine is dependent on pectic enzymes and certain changes in color and flavor are often caused or accelerated by oxidative enzymes; hence, enzymes also are of significance in this industry.

## I. Yeasts

Wine differs from grape must (juice) principally in the fact that the sugars of the must have been converted into  $C_2H_5OH$ ,  $CO_2$  and small amounts of certain by-products of alcoholic fermentation, such as acetic acid, acetaldehyde, several esters, glycerin, lactic acid and succinic acid. Until Pasteur's work and publications on the microbiology of wine (63),

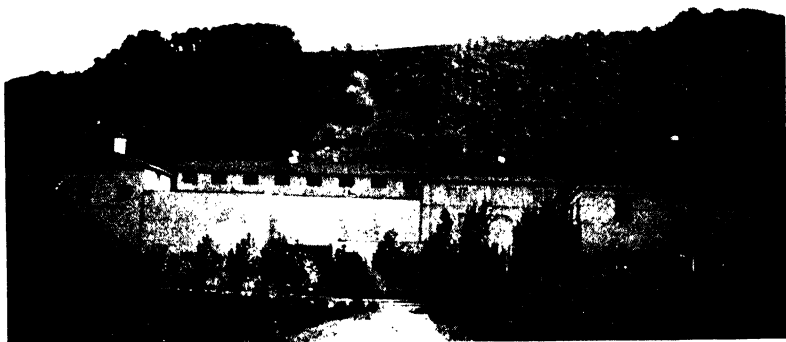


Fig. 1.—A California winery and hillside vineyard.

the nature of alcoholic fermentation was misunderstood, it being a common assumption that the process was nonbiological in nature, or that the yeast always found in fermenting musts developed by spontaneous generation. Pasteur proved that alcoholic fermentation is a biological process induced by yeast and that yeasts do not develop spontaneously out of "thin air." There must be living yeast cells present in the must and from these the yeasts causing the fermentation grow.

It is not possible in the space available in this review to discuss the yeasts of grapes and wine except in a very sketchy manner. Fortunately, there are excellent treatises on the yeasts, such as those of Lodder (49), Stelling-Dekker (76), Jorgensen (39), Guillermond (Tanner) (32), Henrieci (34), Ventre (82), Lafar (47) and others; and many books and papers on the physiology of yeasts and the nature of alcoholic fermentation, such as those

of Harden (33), Rahn (71) and others. We shall attempt to summarize some of the more important.

### 1. *Sequence of Microorganisms in Musts and Wine*

If no measures are taken to favor one group of microorganisms over another, the following events usually take place in grape must.

During about the first 24 hours at room temperature, the so-called wild yeasts, particularly the apiculate yeasts, multiply rapidly and initiate a

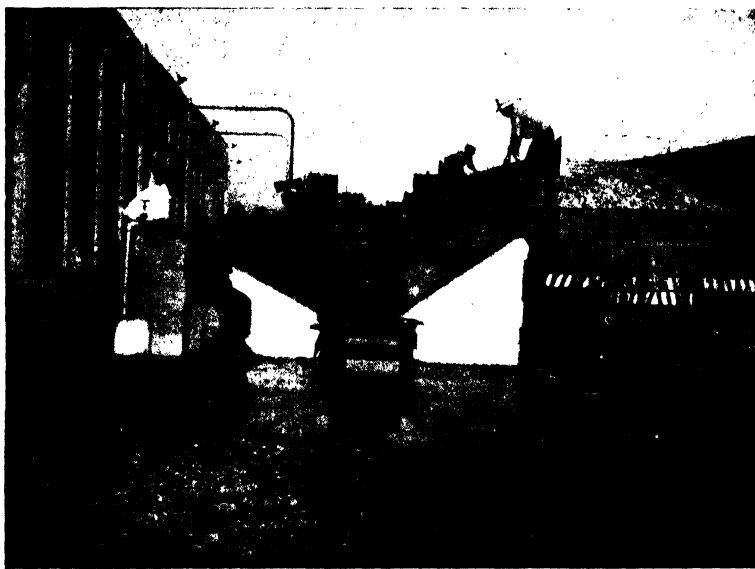


Fig. 2.—Delivering grapes to a California winery.

feeble fermentation; other yeasts of feeble fermenting power such as *Pichia*, *Klöckera* and *Candida* are also often present. Within 48 hours these wild yeasts have been displaced, usually by the true wine yeast, *Saccharomyces ellipsoideus* (which Mrak and others now prefer to term *Sacch. cerevisiae* var. *ellipsoideus*, but which this writer still prefers to designate as *Sacch. ellipsoideus* owing to the long use of this term and the marked differences in alcohol-forming power of typical *Sacch. cerevisiae* and *Sacch. ellipsoideus*). For the ensuing three to four weeks, *Sacch. ellipsoideus* dominates the situation and under favorable conditions will reduce the sugar content from an

original concentration of 15-23 per cent to less than 0.20 per cent with corresponding production of ethyl alcohol and  $\text{CO}_2$ .

If unmolested there will often develop during yeast fermentation one or more strains of *Lactobacillus*, particularly *L. mannitopoeus*, *L. gracilis* or *L. hilgardii*. Also these or other lactobacilli may develop soon after alcoholic fermentation. If left exposed to the air, *Acetobacter* next "takes over" and converts alcohol to acetic acid and  $\text{H}_2\text{O}$ , and transforms the wine into vinegar. We hear much complaint from alleged wine connoisseurs that control of fermentation is very reprehensible and that one should allow Nature to conduct the fermentation and age the wine. Dame Nature's principal aim in this case, however, is to make vinegar, and to be consistent the wine connoisseur should drink vinegar, not wine! Control is essential in the making of a potable beverage from wine, die-hard connoisseurs and hypocritical supporters of "natural wines" (whatever they may be) to the contrary notwithstanding! This writer has little patience with such posers of wine lore and romance.

If left indefinitely exposed to the air, the acetic bacteria will eventually attack and destroy the acetic acid. Then follow molds, and putrefactive bacteria which finally convert most of the remaining organic matter into inorganic compounds of nitrogen and into  $\text{CO}_2$  and  $\text{H}_2\text{O}$  thus completing the carbon and nitrogen cycles.

The responsibility of the enologist and wine maker is to so control conditions that only the true wine yeast is permitted to function and the growth and activity of all other microorganisms (with one possible exception) are prevented. The "one possible exception" is the growth and activity of the malic acid destroying *Micrococcus* species in certain highly acid wines such as those of Switzerland and Germany in which *M. acidovorax* and others destroy the excess malic acid of the new wine and thus render it potable. Most of the important cellar operations in wine making have for their object the control of microorganisms.

## 2. Yeasts Found on Grapes

Pasteur (63) showed that the skins of grapes carry considerable numbers of yeasts and wine disease organisms. Ventre (82) states that during the initial stages of fermentation, apiculate yeasts and "Pastorianus" yeasts are active, followed soon by *Sacch. ellipsoideus*, and that the three groups are readily distinguishable by microscopical appearance. Mrak and McClung (53b), however, indicate that classification on such a basis is not possible and that cultural characteristics including spore formation and

fermentation of various sugars are essential to safe classification. On the other hand, Ventre and other European enologists are probably dealing in generalities when they make such a statement and are using the terms *Sacch. ellipsoideus*, *Pastorianus*, *Apiculatus*, *Torula* and *Mycoderma* in the older sense of general names for ill-defined groups of yeasts.

According to Bioletti, *Saccharomyces ellipsoideus* is the most common true yeast and *Apiculatus* the most common "pseudo yeast" occurring on grapes. Bioletti and Cruess (7) described briefly *Sacch. ellipsoideus*,

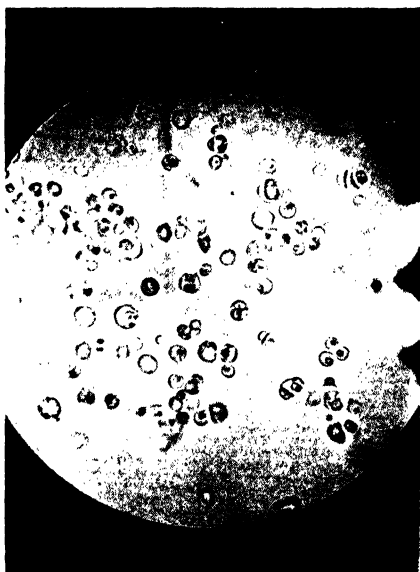


Fig. 3.—*Hansenula Anomala*, variety *spherica*. Found on grapes. Note hat-shaped spores.

*Apiculatus* and several film-forming yeasts isolated from California grapes and wines. Cruess (14) isolated many cultures of yeasts from grapes and fermenting musts and described *Sacch. ellipsoideus*, *Sacch. pastorianus*, *Willia anomalus*, *Sacch. apiculatus*, *Mycoderma* sp. and *Torula* sp. These generic names were in many cases not in conformity with modern terminology and, therefore, the later nomenclature and methods of classification of Mrak and McClung (based on Lodder, Stelling-Dekker and other yeast taxonomists) should be followed. Nevertheless, much of the data reported at that time is of interest and useful to enologists.

For example, it was found that the numbers and character of the micro-organisms present on grapes varied greatly according to the maturity of the grapes. Samples were collected aseptically, crushed aseptically, plated on grape must agar and counted.

On the hard green grapes, molds predominated ranging from about 100 to about 1,000,000 spores per cc. of juice, wild yeasts less than 10 per cc. and *Sacch. ellipsoideus* "none" (*i. e.*, alcoholic fermentation failed to develop in 300-cc. portions of crushed grapes and no *Sacch. ellipsoideus*



Fig. 4.—*Saccharomycodes ludwigii*: is found on grapes. Note spores.

appeared on plates). When the grapes had begun to color, but were still immature, molds still predominated at about 1,000,000 cells per cc. and wild yeasts had increased to about 175,000 per cc. Ripe grapes from these same vines showed about 190 to 22,000 mold spores per cc. of juice, wild yeasts about 3000 to 26,000 per cc. and *Sacch. ellipsoideus* less than 1 per cc., nevertheless present.

On nine samples of grapes as received at the winery, mold spores ranged from 1300 to 9,200,000 per cc., apiculate yeasts 28,000 to 7,341,000 per cc. other wild yeasts 2000 to 8,440,000 per cc. and *Sacch. ellipsoideus*

less than 1 to about 500,000 per cc. A few vinegar bacteria were found in some samples.

These and other observations indicate that wine grapes ordinarily arrive at the cellar heavily contaminated with molds and wild yeasts, but only lightly seeded with *Sacch. ellipsoideus*, the true wine yeast. They also indicate the advisability of using starters of pure wine yeast and of eliminating or controlling the activities of wild yeasts and molds. Cruess (13, 14, 17) and others have demonstrated that the addition of 100-200

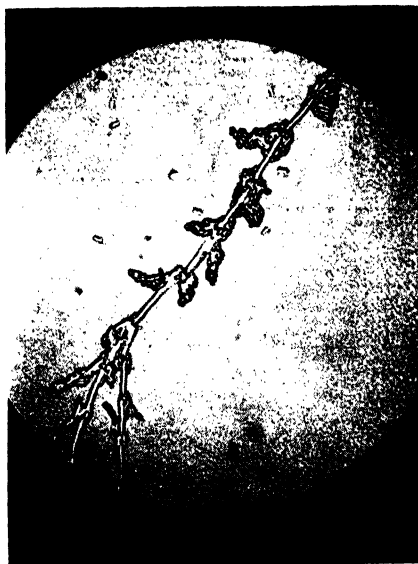


Fig. 5.—*Candida* Species. Is found on grapes. Note pseudomycelium.

p.p.m. of  $\text{SO}_2$  to the crushed grapes or must will effectively inhibit the growth of molds, wild yeasts and bacteria, but allow growth and fermentation by *Sacch. ellipsoideus*; use of  $\text{SO}_2$  is now a practically universal cellar practice in all wine-making regions of the world.

Mrak and McClung (53b) conducted several years' study of yeasts occurring on California grapes and classified the isolated pure cultures much more specifically than had Cruess and other previous investigators. In brief, they isolated and purified 241 cultures from grapes from several localities of the state and on the basis of morphology, cultural characteristics and fermentation properties on various sugars classified them as follows:



*Saccharomyces* (chiefly *Sacch. cerevisiae* var. *ellipsoideus*), 118 cultures; *Zygosaccharomyces*, 13 cultures; *Hanseniaspora*, 11 cultures; *Klöckeriaspora*, 1 culture; *Pichia*, 6 cultures; *Debaromyces*, 3; *Hansenula*, 4; *Zygo-pichia*, 2; *Torulospora*, 1; *Torulopsis*, 25; *Mycoderma*, 6; *Kloeckera*, 16; *Rhodotorula*, 6; *Schizoblastosporion*, 1 culture, and *Candida*, 26 cultures. They indicate that their data agree reasonably well with those of certain European investigators, namely De Rossi (24), Castelli (9) and Verona and Luchetti (81). No definite correlation between the distribution of any genera or species with a particular district was observed. Holm (37), however, found no *Sacch. ellipsoideus* on grapes grown in a new district in Tulare County, California, where wines had never been made. Mrak and McClung were able to allocate most of their cultures to previously named genera and species, although they described several new species, namely, *Torulopsis californicus*, *Torulopsis fermentans* and *Asporomyces wae*. They made no quantitative determinations of the number of cells of the various yeast species present on the grapes.

### 3. Use of Pure Cultures

Most enologists such as Ventre (82), Bioletti and Cruess (7), Bioletti (6), Cruess (13, 17), Pacottet (62), Theron and Nienhaus (77), Müller-Thurgau and Osterwalder (55), Amerine and Joslyn (1) and many others recommend the use of pure cultures of selected wine yeasts in the fermentation of grape musts in order to secure sound fermentations and wines of uniformly good quality.

Important as the use of pure yeast starters is in wine making, it is not as important as the elimination or inhibition of undesirable yeasts and bacteria by use of SO<sub>2</sub>, pasteurization and other means, because there are always present on grapes under commercial conditions sufficient numbers of *Sacch. ellipsoideus* to develop a normal fermentation and produce a wine of good quality provided spoilage organisms are held in check by SO<sub>2</sub> or other effective means.

Nevertheless, some strains of *Sacch. ellipsoideus* do exert a marked effect on flavor and bouquet of wines. In unpublished experiments the writer and D. Guadagni found that among more than 200 pure cultures of *Saccharomyces ellipsoideus* from California grapes, only a few (5 or 6 cultures) markedly affected the flavor and bouquet favorably in comparison with the average "run of the mill" *Sacch. ellipsoideus*. Among the stock cultures from the American Type Culture Collection, Pacottet, Geisenheim and Switzerland in our collection, several yeasts from Germany and Switzerland

gave the most pleasing flavors and bouquets in fermented musts. A fine Chablis-like flavor and bouquet was produced by these and several California yeasts in grape must of neutral flavor.

Therefore, the writer is convinced that it is possible to improve the quality of wine by use of yeasts selected to produce superior flavor and bouquet.

The preparation and application of pure yeast starters is fully described by Amerine and Joslyn (1) in University of California Agriculture Experiment Station Bulletin 639, obtainable from the Publications Office, Agriculture Experiment Station, Berkeley, California.

#### 4. *The Role of SO<sub>2</sub>*

In a subsequent section (on the lactobacilli of wine) is presented briefly the results of observations under commercial conditions of the application of SO<sub>2</sub> and of SO<sub>2</sub> and pure yeast in wine fermentations. In brief, the SO<sub>2</sub> (100–200 p. p. m. added before fermentation) gave wines of low volatile acid content, completely fermented to very low sugar content, and free or practically free of wine disease organisms. Wines in which SO<sub>2</sub> was not used were in most cases high in volatile acid, highly contaminated with wine disease organisms, high in unfermented sugar, and for these reasons very unsound and unstable. Many were unmerchable because of spoilage by lactobacilli notably by *L. hilgardii*.

Porchet (68) and Osterwader (60) report that *Sacch. ellipsoideus* can be acclimated to relatively high concentrations of SO<sub>2</sub>.

#### 5. *Effect of Temperature*

Like other organisms *Sacch. ellipsoideus* exhibits temperature minima, maxima and optima for growth and fermentation. Much research has been done on this factor. Enologists agree that wines fermented at high temperatures, 30–40° C., are of poorer flavor and bouquet than those fermented at more favorable temperatures for quality such as 15° C. to 25° C.

At temperatures above 35° C. the fermentations are apt to "stick," cease with considerable unfermented sugar remaining. At temperatures below 10° C. fermentation is very slow and may also "stick."

Wines that stick at elevated temperatures are apt to be very unsound and often develop bacterial spoilage later in storage.

During industrial fermentation of grape must, much heat is liberated by the conversion of sugars to C<sub>2</sub>H<sub>5</sub>OH and CO<sub>2</sub> and the temperature if un-

controlled may rise to 35–40° C. and the fermentation will “stick.” Hence, it is customary to cool artificially the must during fermentation.

Hohl and Cruess (35), using syruiped fermentations found that *Sacch. ellipsoideus*, so-called Champagne strain, formed at 7° C. 16.45% alcohol by volume, at 10° C. 16.4%, at 16° C. 16.65%, at 20–22° C. 16.5%,

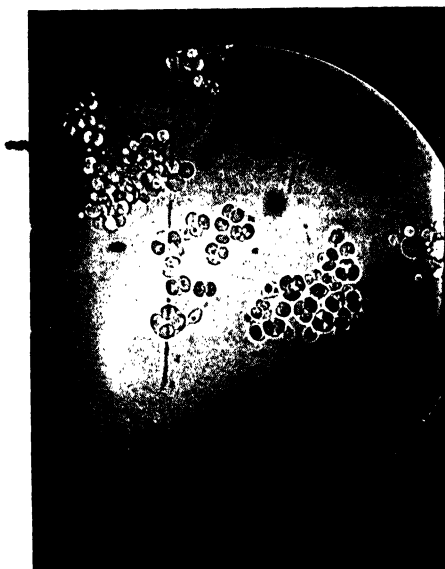


Fig. 6.—*Zygosaccharomyces* sp., showing spores and conjugation. Note spores. (Photographs of Figs. 3–6 by H. J. Phaff.)

at 25° C. 13.9%, at 28° C. 13.3%, at 31° C. 12.2%, at 34° C. 8.6% and at 37° C. 6.35%. Müller-Thurgau has reported similar data.

Osterwalder (61) and Porchet (67) report on yeasts adapted to fermentation at low temperatures near 0° C. for use in Swiss wine cellars.

#### 6. Effect of Acetic Acid on Yeast

Acetic acid is toxic to yeast even in relatively low concentrations. Cruess and Hascal (19) found that 0.30 gm. of acetic acid per 100 cc. greatly retarded yeast growth and activity and 1.0% completely prevented growth of *Sacch. ellipsoideus*. However, Cruess and Gililand

(unpublished data) find that some yeasts grow in pickles and on pickle brines at 1% acetic acid. See also Cruess, Richert and Irish (21) in which it is reported that the toxicity of acetates varies inversely with the pH value, the lower the pH value the more toxic to yeast is the acetic acid. Evidently the undissociated acid is the toxic agent. Porchet (69) has recorded observations on the effect of acetic acid on fermentation.

### 7. *Alcohol-Forming Ability*

By normal fermentation of grape must of high sugar content it has been usually assumed that *Sacch. ellipsoideus* will form a maximum of about 16% of alcohol by volume. Cruess (14), reported a maximum alcohol production of 15.6% by several strains of *Sacch. ellipsoideus* from California grapes. Hohl and Cruess (36), however, encountered considerably higher alcohol-forming power among twelve strains of *Saccharomyces* and *Torulopsis* isolated from Spanish Sherry and French Arbois wine films. The alcohol attained by these yeasts in 30° Brix must ranged from 15.6 to 18% alcohol by volume. Sannino (72) gives the usual maximum at 16.0–16.5% by volume.

In other experiments made in this laboratory (unpublished data) the laboratory's Burgundy and Champagne strains of *Sacch. ellipsoideus* have rarely produced above 16% alcohol by normal fermentation. By syruiped fermentation they have attained 18% or more. (See next section.)

### 8. *Syruped Fermentation*

Fortified or so-called dessert wines are fortified with high proof brandy to 20–21% alcohol. A tax must be paid on the alcohol used and the added alcohol markedly dilutes the flavor of the wine. In 1916 Cruess, Brown and Flossfeder (18) reported on a new method of producing wines of high alcohol content in which grape concentrate of about 70° Brix is added repeatedly in small amounts toward the end of fermentation. By this method wines of 18–20% alcohol were obtained. The so-called Burgundy yeast strain was used.

Hohl and Cruess (35) obtained by similar means wines of 17.06 to 18.05% alcohol. Grape concentrate proved superior to cane sugar and dextrose for these additions, consequently it would appear that the grape concentrate in some manner stimulates the yeast to high alcohol production. However, pure ascorbic acid and pure thiamin chloride failed to cause a similar effect.

More recently, using their Spanish Sherry and French Arbois yeasts, Hohl and Cruess (36) produced wines of 17.6–19.0% alcohol by syruiped fermentation.

In a commercial experiment with 4000 gallons of must, there was obtained by syruiped fermentation nearly 18% alcohol (Cruess unpublished data, 1936).

It is believed that this method has commercial possibilities as it produces

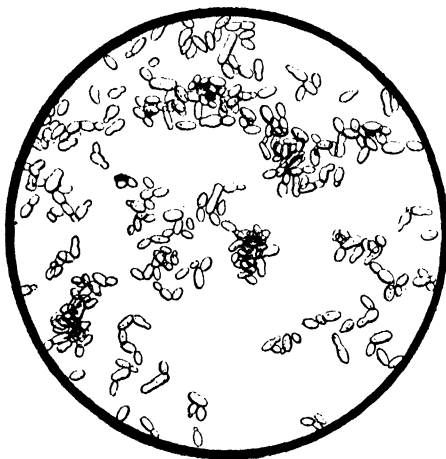


Fig. 7.—Pasteur's *Mycoderma vini*.

wines of richer flavor than those obtained by the usual fortification-with-brandy procedure.

#### 9. Sherry and Arbois Film Yeast

Pasteur (63) many years ago observed that the film yeast which developed on the surface of wine in purposely partially filled casks in the Arbois district (Chateau Chalon, etc.) aided in producing the characteristic "yellow wines" ("vin jaune") of that district. He called the yeast *Mycoderma vini*.

In Jerez de la Frontera, Spanish sherries are aged under a similar yeast film, long assumed to be *Mycoderma vini*. The Spanish call it "flor" (flower) and the process is called flowering in France and Spain.

Acetaldehyde and esters are formed and the wine acquires a very char-

acteristic, rather pungent bouquet and flavor with a slightly bitter after-taste.

Often, however, *Acetobacter* (Pasteur's *Mycoderma aceti*) gains the upper hand and converts the wine into vinegar.

Prostdosserdow and Afrikian (70) (reference through Schanderl) evidently were the first to show that some of the yeasts from Jerez wine films form spores and form high alcohol content in musts and should, therefore, be classified not as *Mycoderma* since the latter forms no alcohol. Schanderl (73) studied the Jerez yeasts further, also finding that the film stage developed normally in pure cultures after these yeasts had fermented the sugar of the must; also that they formed spores, and closely resembled *Sacch. ellipsoideus*.

Hohl and Cruess (36), as previously indicated, studied twelve bottom fermentation yeasts and three nonfermenting film yeasts from Arbois wines and Jerez wines. The twelve strongly fermenting yeasts also readily developed films on wine; at 15.4–16.4% alcohol, the alcohol tolerance varying somewhat with the strain. Under practical conditions we have succeeded in securing film growth on wines of 17% alcohol content by volume. High tolerance to alcohol is a very valuable property since vinegar bacteria seldom grow at above 15% alcohol.

As previously mentioned these yeasts exhibited very high alcohol forming power, 16.0–17.7% by volume, compared with 15.6% by volume formed by our so-called Champagne strain in parallel experiments.

Several of the yeasts were found to form spores and were classified as *Sacch. cerevisiae* var. *ellipsoideus*; several others of high fermenting power failed to form spores and so were classified as *Torulopsis*.

These film yeasts, both the Jerez and the Arbois strains, are now in commercial use in California in the production of Spanish type sherries. Three *Pichia* yeasts and one *Hansenula* yeast were recovered from the naturally occurring film stage, but because of low alcohol tolerance, 13% or less, were not considered of significance.

The film stage of the Jerez and Chalon yeasts oxidizes ethyl alcohol to acetaldehyde or to CO<sub>2</sub> and H<sub>2</sub>O and also oxidizes acetic acid and the fixed acids of the wine. Pasteur had noted the destruction of acetic acid in wine by the Arbois wine film yeast. Cruess and Podgorny (20) have reported on this effect in wine also.

The Jerez and Arbois yeasts form a very interesting group. We have been unable to recover similar yeasts from California wines although Schanderl (73) reports success with German yeasts and Niehaus (58) found a number of such film-forming yeasts in South African wines.

## II. Microbial Spoilage of Wine

Wine, like other liquids of biological origin, is subject to spoilage by various microorganisms, although until Pasteur's (63) classic researches on the spoilage of beer and of wine, it was not generally recognized that spoilage is often due to these agencies. For example, Chaptal (10) and others of his era stated that vinous fermentation is due to the interaction between the sugar principle and protein or albuminous substances of the grape must (juice). If the two are properly balanced the sugar and albuminous substances are both removed and a sound wine results. If the albuminous substances are more abundant than the sugar, then they are capable of bringing about all the diseases to which wine is susceptible. It was also believed even in the time of Pasteur according to Ventre (83) that if microorganisms appeared in wine and caused its spoilage, they, like the yeasts which caused fermentation of the juice to wine, were spontaneously generated from the "glutenous substances" of the wine. In other words, the nonbacterial theory of wine spoilage died hard. However, in addition to being a brilliant investigator, Pasteur was also a past master in defending and promoting his new findings and theories. Consequently not many years elapsed before his views were accepted and most types of wine spoilage were recognized as microbial in nature.

Not all wine spoilage is caused by microorganisms, however. Thus an excess of iron, copper or tin may cause clouding of wine and severe oxidation may ruin it for beverage purposes. Yet in the main, wine "diseases" are usually caused by microorganisms and usually by bacteria rather than by yeast or molds. Nevertheless, aerobic film yeasts (Pasteur's *Mycoderma vini*) and sediments forming facultative yeasts occasionally cause deterioration of wine. Also, the bacterial spoilage of wine can be caused by aerobic bacteria (acetic bacteria), or by facultative bacteria such as certain lactobacilli and certain coccus forms.

### 1. Spoilage by Film-Forming Yeasts

Many of the wines of the Arbois region of France (Pasteur's "home town") in Pasteur's time as well as today were aged aerobically in partially filled casks under a grayish film or "fleur du vin," which Pasteur proved to be composed of yeast-like cells. He named this organism *Mycoderma vini*. While this film yeast used in this manner is beneficial and necessary to attainment of the well-known, pungent flavor and bouquet of Arbois and Chalon wines, it can also under other circumstances spoil certain wines

by destroying much of the fixed acid and alcohol and by imparting a disagreeable flavor and odor. The use of these film yeasts in preparing Chalon and Jerez (sherry) wines is discussed more fully in a separate section.

Pasteur grouped all of the film yeasts of wine under the name *Mycoderma vini*. Later this term came to be applied only to nonsporulating, non-fermenting film yeasts. The work of Prostdosserdow and Afrikian (70), Schanderl (73), Hohl and Cruess (36), and others has shown that usually the desirable Arbois, Chalon and Jerez film yeasts are vigorous fermenters of sugary liquids and that the majority of them form endospores.

Hohl and Cruess (36) recovered from Jerez wine and Chalon wine "mycoderma" films four "wild yeasts," namely, three *Pichia* forms and one *Hansenula*, all of which formed spores but little or no alcohol. They were not very tolerant to alcohol, and formed films at 11.2% for the least tolerant to 13.7% alcohol for the one most tolerant to alcohol, in contrast with the desirable Chalon and Jerez film formers which in some cases tolerate up to 17% or more alcohol.

The *Hansenula* and *Pichia* forms are common film formers on "distilling material" (diluted wines used for distillation for brandy). They grow profusely on such wines wash-stored for a few days in open vats awaiting distillation. It is likely that they destroy some alcohol by oxidation to CO<sub>2</sub> and H<sub>2</sub>O and impart off flavors under such conditions. To the extent to which these changes occur their development under these circumstances is undesirable.

"*Mycoderma*," using this term in the Pasteurian sense, is never encountered on fortified wines of 18–22% alcohol even if stored in open vats, as the alcohol prevents their growth. It seldom develops also on unfortified ("natural") California wines unless they have been diluted to low or moderate alcohol, *i. e.*, below 13% probably, even in partially filled containers. Vinegar bacteria are more apt to appear. However, in Switzerland, Germany and France where many natural wines may contain as little as 10.5% or even less of alcohol, film yeasts can grow readily if the air supply is adequate.

These yeasts at first usually outgrow the vinegar bacteria, but eventually the grayish-white yeast film gives way to the translucent, tough, shiny acetic bacterial film, or to the "non-film" forming acetic bacteria. During the film yeast stage, the total acid decreases by oxidation by the yeast and during the acetic stage it increases owing to acetification.

Spoilage of wine by either film yeasts or vinegar bacteria is evidence of gross negligence on the part of the wine maker. If the casks and bottles



are at all times full and sealed these microorganisms cannot develop or cause spoilage.

**Clouding and Sedimentation Caused by Yeast.**—It has long been recognized in France, California and in other wine-producing regions that sweet Sauternes and Sauterne type wines are subject to fermentation with accompanying clouding by wine yeasts *Sacch. ellipsoideus*, unless the wines are “pickled” with SO<sub>2</sub> (300–400 p. p. m. of SO<sub>2</sub>) to prevent yeast growth, or are pasteurized, or “germ proof” filtered to render them sterile.

Baker (3) in this laboratory has isolated a number of cultures from bottled California Sauterne wines that had begun to ferment in the bottles at about 13% alcohol and 2 to 8% residual sugar. In such cases the yeasts were usually of true wine yeasts, *Sacch. ellipsoideus* (synonymous with *Sacch. cerevisiae ellipsoideus*). In such cases the causative organism is easily identified and control measures are simple. Baker found that some of these yeasts will tolerate more than 400 p. p. m. of SO<sub>2</sub> indicating that the customary legal maximum of 300 p. p. m. is inadequate in many cases. The practical observations of wine bottlers confirm his observations. Hence, in California it is customary to elevate the SO<sub>2</sub> content at the time of bottling to 350–400 p. p. m. (350–400 mg. per liter).

But in addition to this common form of spoilage, dry wines such as Chablis or Riesling types of even less than 0.15% residual sugar will, according to Cruess (12), Phaff and Douglas (64), occasionally become cloudy or show a white deposit brought about by yeast. In some cases, also, fortified sweet wines such as muscatel, port and angelica of 20% or more alcohol by volume exhibit yeast clouding and sedimentation.

## 2. Spoilage by Vinegar Bacteria

Since time immemorial the souring of wine by vinegar bacteria has been a well-known and very common phenomenon; the makeup of the word vinegar from “vin aigre,” sour wine, is evidence of this fact.

As is true of several other common forms of wine spoilage, Pasteur (63) showed that acetification of wine is due not to some mysterious purely chemical transformation or by spontaneously generated organisms, but to the activity of what he termed *Mycoderma aceti* or acetic bacteria. He found it associated often with film yeast, his *Mycoderma vini*, and that more often than not the *Mycoderma aceti* outgrew the *Mycoderma vini* and eventually transformed the wine into vinegar. Incidentally he also showed that vinegar making can be greatly hastened and improved by use of a new

technique in which only a portion of each barrel of vinegar is drawn off for bottling and this portion is replaced with wine. This procedure gave the newly added wine a tremendous inoculation or starter of vinegar bacteria which rapidly converted the added wine to vinegar, and at the same time maintained constancy of quality.

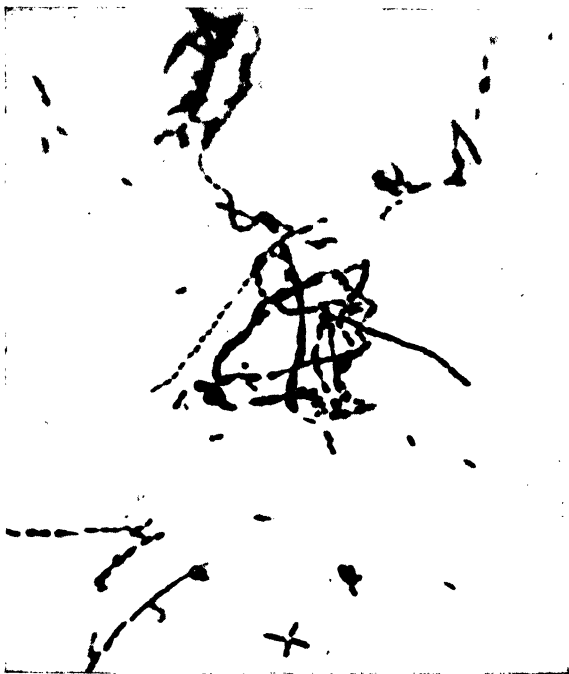
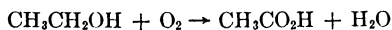


Fig. 8.—*Acetobacter*, involution forms. (After Vaughn, in Wallerstein Communications, 1942.)

Vinegar bacteria, of course, require oxygen for growth and acetification, *i. e.*, transformation of ethyl alcohol into acetic acid:



Therefore, all that the wine maker need do to prevent acetification of his wines is to exclude oxygen by keeping his casks and bottles well filled and tightly bunged or corked. In fact, that practice is Rule No. 1 of cellar practice! Nevertheless, even in the best of cellars, casks will occasionally

leak, thus developing a head space and admitting air, or corks may become loose and admit air. In both cases acetification is very apt to follow unless the wine is high in alcohol or contains sufficient  $\text{SO}_2$  to inhibit growth. Cruess (13) showed that vinegar bacteria are sensitive to  $\text{SO}_2$  in must and more recently Cruess and Podgorny (20) showed that a relatively small concentration of  $\text{SO}_2$  inhibits their growth in wine. (See also Cruess, Weast and Gililand (22).)

The tolerance of acetic acid bacteria for alcohol appears to be quite variable, judging from the experience of Cruess, Weast and Gililand (22). They found that in most cases naturally occurring acetic bacteria of wine did not grow in wine of 14.5–15% alcohol, although growth and acetification occurred under winery conditions at 14.9% alcohol. Vaughn (78) states that the limit for growth of acetic bacteria is considered to lie between 14 and 15% alcohol. Bioletti (5) placed the maximum tolerance of vinegar bacteria at 14% alcohol.

Often, particularly in fermentation in which little or no  $\text{SO}_2$  has been used, acetic bacteria develop in the floating cap of skins and stems in red wine vats and cause considerable rise in volatile acidity of the wine.

In the hot interior of San Joaquin Valley of California at one time, 1933 to about 1936, losses of fermenting grape must of the Muscat variety were quite heavy owing to acetification during alcoholic fermentation. Vaughn (79) proved that the rapid acetification was due to the activity of vinegar bacteria capable of growth and acetification in the presence of actively fermenting wine yeast, *Saccharomyces cerevisiae* var. *ellipsoideus*. Cruess and Hascal (19) found that vinegar bacteria added in generous amount to grape must with a starter of wine yeast in some cases caused cessation of alcoholic fermentation by acetic acid. Similar results were reported by Cruess (16) in which it was found that vinegar bacteria can arrest alcoholic fermentation by yeast by creating sufficient acetic acid to arrest yeast activity.

Vaughn (79) found that not all strains of *Acetobacter* possessed the power of rapid acetification in association with yeasts. However, all cultures isolated by him from the acetified Muscat musts, previously mentioned, possessed this property to a very marked degree. Acetification was more rapid and extensive in the presence of yeast at 37° C. than at 31° C. In commercial practice 37° C. is often attained in the crushed grapes in the hot San Joaquin Valley wine area. In sterile grape must the vinegar bacteria isolated from the affected Muscat wines produced a mousey taste and odor, a characteristic usually associated with lactobacilli.

Prevention of this form of spoilage during alcoholic fermentation is easily

accomplished by adding 100 p. p. m. of  $\text{SO}_2$  or an equivalent amount of bisulfite to the must before fermentation.

Vaughn (78) has recently presented a very useful résumé of the properties and industrial application of the acetic bacteria and among other properties mentions their ability to oxidize a considerable number of organic compounds other than ethyl alcohol. For example, *d*-sorbitol is oxidized to *l*-sorbose useful in the synthesis of vitamin C. *d*-Glucose is oxidized by *Acetobacter aceti*. He also discusses the classification of the acetic bacteria and favors the name *Acetobacter* for the genus in preference to *Bacterium*, *Mycoderma*, *Bacillus* and several other generic names used by others. He suggests that they belong in the family *Pseudomonadaceae* as proposed by Kluyver and van Niel in 1936. The type species is *Acetobacter aceti* (Kützing) Beyerinck. Vaughn points out that the genus has been encumbered with a large number of species, many of which are no longer recognizable. Others are probably synonymous with previously described species.

Some species form a heavy pellicle on wine during acetification; others form no pellicle or "mother," yet bring about rapid acetification. *Acetobacter* does not convert glucose directly into significant amounts of acetic acid. Vaughn emphasizes that different species have no specific environment; a given species may be found in beer, wine, cider, perry, etc. He lists seven species based on nature of film, cellulose reaction, optimum temperature, oxidative characteristics, pigment formation, ketogenic properties and ability to use ammonium salts as sole source of N. These species are *A. aceti*, *A. xylinum*, *A. rancens*, *A. melanogenum*, *A. roseum*, *A. suboxydans* and *A. oxydans*. All are strongly aerobic in respect to  $\text{O}_2$  requirements.

Finally, it may be said that vinegar bacteria often spoil wine for use as such; yet in so doing they do not destroy its commercial value completely because the wine maker can convert the spoiled wine into wine vinegar, a merchantable commodity.

### 3. Spoilage by Certain Lactic Bacteria

The most serious losses by wine makers through bacterial spoilage are caused by rod bacteria most of which belong to species of *Lactobacillus* and are facultatively aerobic in character. In the wine industry such spoilage is termed the "tourne disease" or "lactic souring." In France if it is accompanied by gas formation it is termed "pousse," although the term "pousse" may occasionally be applied also to a desirable bacterial transformation by *Micrococcus acidovorax* in which gas is also produced.

In typical "tourne" spoilage the wine shows a silky "sheen" or silky cloudiness when the bottle or glass is gently agitated and held toward the light. It also is increased markedly in volatile and in fixed acidity and develops a disagreeable "lactic sour" taste or in some cases a distinctly mousey taste and odor. Under the microscope it will be found teeming with long rod bacteria, typical microscopical fields of which are very well portrayed in Pasteur's paper, "Études sur le vin," 1873. Very often in California development of the typical "mousey" "milk-sour" flavor of "tourne" is accompanied by gas production ( $\text{CO}_2$ ). Some French writers place such wines in a separate category and call the disease "pousse," owing

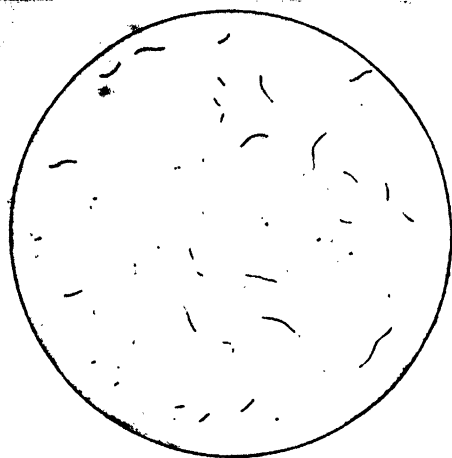


Fig. 9.—Bacteria from wine. (After Pasteur.)

to the gas pressure in casks or bottles. Pasteur was inclined to consider "tourne" and "pousse" a single disease, "tourne-pousse." Müller-Thurgau and Osterwalder also state that there is no clear-cut differentiation, as the organisms responsible for the two conditions are very similar and in some cases a single organism, as *L. mannitolopoeus* can cause both diseases. Duclaux (28) is also in favor of combining the two diseases as one. Semichon (75) joins in Duclaux's opinion. Kayser (40), Dugast (29a), Pacotet (62) and Couche (11) separate the two diseases.

While Pasteur definitely proved that such spoilage is due to the action of these long rod or filamentous lactic and volatile (acetic) acid-producing bacteria, he did not isolate the organisms in pure culture.

In commercial practice it has long been recognized that these bacteria are much more apt to develop in wines containing considerable residual, unfermented sugar, *i. e.*, wines in which the yeast fermentation has "stuck" because of high temperature or competition with bacteria. In France it was proved more than thirty years ago by several enologists and in California by Bioletti and Cruess (7), Cruess (16) and confirmed more recently by Cruess (15) that by the addition of moderate concentrations of  $\text{SO}_2$  or of bisulfites to the grape must before fermentation, much sounder wines of lower initial volatile acidity, lower residual sugar content and much better keeping quality are obtained. In a typical case one California winery in the first year following repeal produced about 200,000 gallons of wine by "natural" fermentation without use of  $\text{SO}_2$ , all of which became affected with "tourne" and had to be sold at low price and great financial loss for

TABLE I

EFFECT OF  $\text{SO}_2$  IN FERMENTATION ON COMPOSITION AND SOUNDNESS OF RESULTING COMMERCIALY PREPARED WINES. (AFTER CRUESS, (15))

Method of fermentation	Number of samples	Volatile acid as grams per 100 cc.	Residual sugar grams per 100 cc.	Total acid grams per 100 cc.	Alcohol volume, %
1. Natural. No $\text{SO}_2$	51	0.137	0.566	0.51	12.93
2. $\text{SO}_2$ used	98	0.043	0.150	0.66	12.46

distilling material. In the following year  $\text{SO}_2$  was used and as a result the entire output was sound and salable as good wine. In the above table are given a few data on the effect of  $\text{SO}_2$  on composition and soundness of wine. Any new wine containing more than 0.10 gm. of volatile acid per 100 cc. is considered unsound. It should not exceed 0.06 gm. as a matter of fact.

The new wines in which no  $\text{SO}_2$  was used in fermentation showed many millions of long rod bacteria per cc., whereas those in which  $\text{SO}_2$  had been used during fermentation were free or practically free of such organisms. In neither group of wines were vinegar bacteria evident in most cases. The high volatile acidity evidently had been caused by the "tourne" bacteria.

These organisms, however, do not confine their activity to the period of alcohol fermentation. In fact, they very often make their first appearance several months after the alcoholic fermentation has ended. During the increase in temperature of the wine in Spring following the previous vintage the new wine is apt to become cloudy, and exhibit evolution of  $\text{CO}_2$ . If

the disease is not arrested, the wine rapidly spoils. Pasteurization and addition of  $\text{SO}_2$  are the common remedial measures.

It is believed by most investigators in this field—for example, Müller-Thurgau and Osterwalder—that the condition known as “tourne disease” can be caused by any one of several closely related, long rod bacteria.

By regular monthly examination of each tank of wine in the cellar, by analysis for volatile acid content and microscopical examination of the centrifuged sediment for presence of long rod bacteria, the winery chemist can detect incipient development of the tourne disease organisms. An appreciable increase in volatile acidity should cause him to become suspicious



Fig. 10.—Pasteur's Tourne Bacteria.

of the development of tourne organisms, but such suspicion should be confirmed by microscopical examination.

Kramer (43) considered the tourne disease a condition in which bacteria first split the proteins of the wine into amino acids and then decomposed the organic acids with formation of formic, propionic, acetic, succinic, butyric and lactic acids. But Müller-Thurgau and Osterwalder believe that Kramer was dealing with a badly decomposed wine in this case, particularly since butyric acid is not encountered in simple tourne wines, according to them. Duclaux (28) emphasized that tartrates decrease rapidly and volatile acid increases. Much of the volatile acidity he found to be due to propionic acid.

Laborde (45) recovered a culture of bacteria from tourne wine with which he was able to produce the disease in a wine inoculated with it. It caused a decrease in tartrates and produced acetic and propionic acids. It produced mannite from levulose, an attribute also of *Lactobacillus mannitopoeus* of Müller-Thurgau and Osterwalder.

Pasteur (63) and others based the term "tourne" as much on the "turning" in color of affected red wines as on the turning lactic sour. Affected wines on exposure to air, according to Pasteur, turn brown and precipitate their color.

Mazé and Pacottet (52a) reported long, filamentous rod bacteria in old tourne wines and that the organisms form mannite as well as acetic and lactic acids; their description closely parallels that of the "ferment mannitique" of some French enologists and that of the *Lactobacillus mannitopoeus* of Müller-Thurgau and Osterwalder (55). The latter report that "tourne" wines are encountered in Switzerland although less common there than in Southern France and North Africa owing to the inhibiting effect of the high fixed acidity of Swiss wines. Low acidity favors "tourne," as does also residual unfermented sugar. Müller-Thurgau and Osterwalder comment on the "astonishing" absence of information on "tourne" in German publications and complete ignoring of the work of Gayon, Dubourg, Semichon and other French scientists on this disease, possibly because the disease has been very uncommon in the highly acid German wines. Perhaps, also, the common use of SO<sub>2</sub> in German wines has held the "tourne" organisms in check.

The European papers on "tourne" indicate the variability of the symptoms and the fact that the disease is not caused by any one organism. Müller-Thurgau and Osterwalder, however, in their laboratory at Wädenswil, Switzerland, in 1912, brought order out of previous confusion by isolating pure cultures of the responsible bacteria, studying their morphological and physiological characteristics, and reproducing the various "lactic" diseases with the pure cultures. We shall discuss their work in a later section.

Niehaus (56, 57) reports a long, threadlike bacterium from sweet, fortified, South African wines capable of growing actively at 18% alcohol. It required a growth-promoting substance from yeast sediment and a moderate amount of sugar. It formed mannite from levulose. Fevrier (30) found a long, rodlike organism responsible for the spoilage of fortified South African wines of 19–20% alcohol content. It was extremely heat resistant withstanding 80° C. for 15 min. Pacottet and other European authors had placed the death temperature of the "tourne" organism at



about 60° C. in 1 to 2 min. The present author (Cruess) has encountered similar organisms in fortified California wines of 20% alcohol content, which imparted a mousey taste to the wines and in the impure, natural state in the wine withstood 80° C. for over 2 min.

d'Estivaux (29b) found a long rod bacillus in spoiled, fortified Port wines from Portugal growing readily at 18.1% alcohol. It was gram-positive and often appeared in pairs of cells forming an obtuse angle. This (obtuse angle pairs of cells) is a common characteristic of the long rod bacteria found in lactic soured, "tourne," "pousse" and mannitic wines.

Pacottet (62) reported that "tourne" was common in Argentinian wines at the time of his visit to that country about 1910.

Pederson (65) has published a very useful bulletin on the lactic bacteria including those from wine.

In 1936 Douglas and Cruess reported on a study made by them of the "tourne" disease of California wines and described the responsible micro-organism. The disease usually appears soon after fermentation in "stuck wines" (those in which alcoholic fermentation has ceased because of excessive temperatures during fermentation), sometimes soon after alcoholic fermentation has ceased, but more often in early Spring with rise of temperature at that time. The volatile acidity rises rapidly, often reaching 0.30 gm. per 100 cc. A silky cloudiness develops. Eventually the odor and flavor are adversely affected, "mousiness" best describing this condition. Its growth is prevented by the presence of 75-100 p. p. m. of SO<sub>2</sub>, and this is the common preventive measure. If the disease has appeared and has not yet produced a mousey flavor and odor, it can be "cured" by flash pasteurization at 80° C., close filtration of the pasteurized wine, and occasional additions of SO<sub>2</sub> or bisulfite to maintain a concentration of above 75 p. p. m. of SO<sub>2</sub>. Use of 150 or more p. p. m. of SO<sub>2</sub> during fermentation usually gives in the new wine sufficient SO<sub>2</sub> to prevent bacterial spoilage for several months.

Pure cultures from more than twenty wines were secured. Two cultures failed to produce the disease in wine; all of the others produced it readily in susceptible wines and were apparently of a single species. The cells were nonmotile, nonsporulating rods, occurring singly or in pairs, or in short chains of three or four cells, and often in pairs of cells forming an angle of 90-120°. The average range in size of the cells was  $0.9 \times 4.5$  —  $0.9 \times 6.5 \mu$ . The organism was gram-positive. It was isolated on grape juice agar anaerobically, but after several transfers grew readily aerobically. In diluted, sterile, sweet wine (Muscatel, Port, etc.) of 10-12% alcohol, it grew abundantly in 48-72 hrs. at 30-33° C.; it also grew rapidly

in diluted California grape juice. In pure culture the characteristic mousey odor and flavor developed. It fermented levulose, dextrose and xylose, forming acid but no gas, final  $pH$  being about  $pH$  4.0. No fermentation was observed in arabinose, mannose, mannite, glycerol, lactose, sucrose, galactose, raffinose and dextrin. The principal end products of levulose fermentation were lactic and acetic acids. No mannite was found. Growth was inhibited by 75 p. p. m. of  $SO_2$ . Maximum alcohol tolerance



Fig. 11.—Jerez film yeast growing on wine.

for fairly rapid growth in  $SO_2$ -free wine reinforced with 4.5 cc. of grape concentrate per 100 cc. was 16.2% by volume, although slight growth occurred in two months at 18% alcohol. Since it did not form  $CO_2$  in culture media or in wine, it was not a "pousse" disease organism; also for the same reason it would be classed as a homofermentative lactobacillus rather than a heterofermentative one. It was considered a new species and named *Lactobacillus hilgardii* in honor of Dr. E. W. Hilgard, founder of the College of Agriculture of the University of California and the first to

organize wine research in California. Douglas and Vaughn (unpublished) consider this organism very close to *Lactobacillus plantarum* in characteristics and plan to redescribe it in the light of recent new data.

#### 4. *Pousse*

As previously stated, the so-called "pousse" disease of wine differs from the "tourne" disease chiefly in the fact that gas is formed and usually mannite. Perhaps it should be classified as a "vin mannitique" disease. Pacottet's and other previously mentioned views (that "tourne" and "pousse" are separate manifestations of a single disease) are not borne out by the work of Douglas and Cruess as reported in the preceding section. Their *L. hilgardii* formed no gas or mannite in pure culture, yet reproduced in susceptible wine the other characteristics of the "tourne" disease.

As Müller-Thurgau and Osterwalder (55) point out, one aspect of the "pousse" condition (gas pressure in cask or bottle) can also be caused by *M. acidovorax*, a beneficial organism occurring almost universally in Swiss wines.

#### 5. *Mannitic Disease of Wine*

If one allows a drop of lactic soured, spoiled wine to evaporate spontaneously on a microscope slide at room temperature, very likely there will be observed under the low power of a microscope typical rosettes of mannite crystals,  $C_6H_8(OH)_6$ , the hexahydric alcohol of levulose. The author has frequently made this observation on California wines that have "stuck" with some unfermented sugar owing to high temperatures during fermentation. European and other investigators, of course, noted this condition many years ago and have described it and the microorganisms quite fully. Pacottet (62), Sannino (72), Niehaus (56), Semichon (75), Laborde (46), Couche (11), Gayon and Dubourg (31), Müller-Thurgau and Osterwalder (55) and many others have referred to it in their books and research papers. The common view among them until the work of the Swiss investigators (Müller-Thurgau and Osterwalder (55)) had been that the disease develops chiefly during alcoholic fermentation, particularly in hot localities as in North Africa and in Southern France. They showed that the mannite-forming bacteria grow readily in susceptible wines after alcoholic fermentation has ceased.

Gayon and Dubourg (31) isolated pure cultures from affected wines and studied their properties in considerable detail. While some of their observations and conclusions do not agree with those of Müller-Thurgau and

Osterwalder (55) on the latter's *Lactobacillus mannitopoeus*, nevertheless Gayon and Dubourg's (31) papers (1894 and 1901) are very useful and informative Mannite was formed from levulose only Some  $\text{CO}_2$ , fixed acid

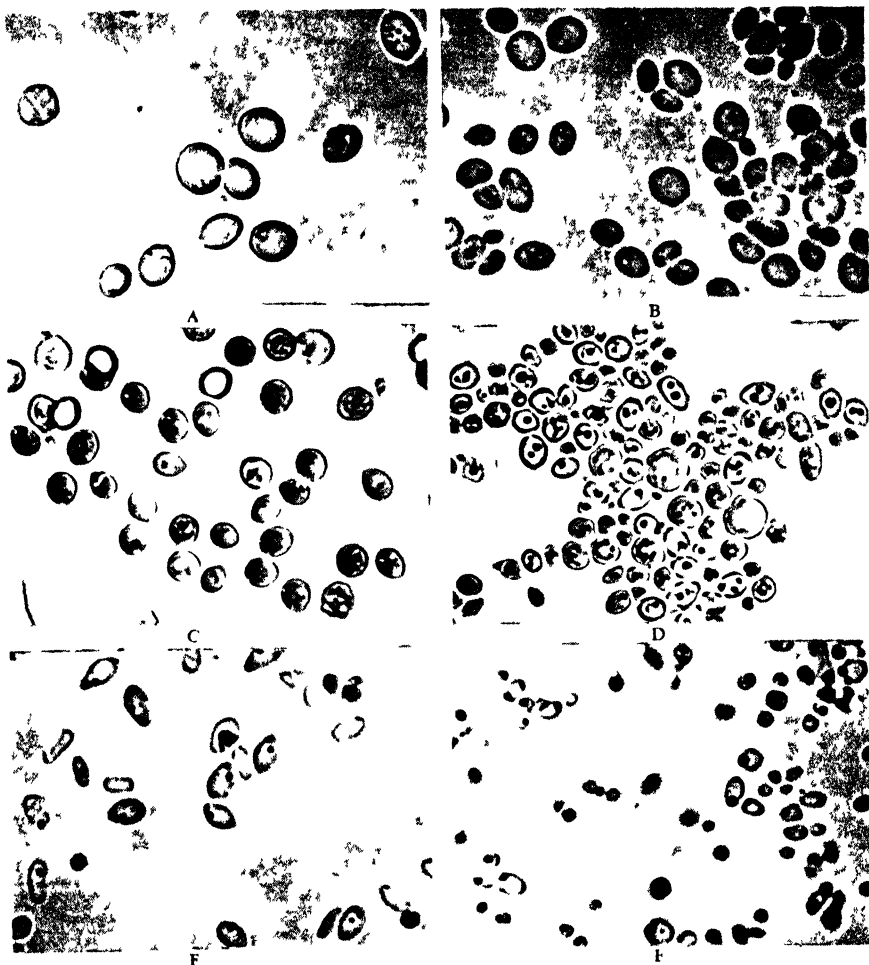


Fig 12—Photomicrographs of typical members (A) Jerez yeast 5 (*Torulopsis*) fermentation stage, 5 days old (B) Jerez yeast 5, film stage, 2½ months old (C) Chalon yeast 12 (*Saccharomyces cerevisiae*, *ellipsoideus*), fermentation stage, 5 days old (D) Chalon yeast 12, film stage, 2½ months old (E) 5 days old film of yeast 1, (*Pichia*) (F) 5 days old film of yeast H a (*Hansenula saturnus*) (Hohl and Cruess (36))

and considerable volatile acid were formed simultaneously with the mannite. The fixed acid produced from levulose or dextrose was chiefly lactic and the volatile almost wholly acetic. The organism did not attack tartrates and in this respect, they state, differed from the *tourne* organism, which destroys tartrates. They report that their culture also formed alcohol, glycerol and succinic acid. The mannite formed by the bacteria is not destroyed, consequently it remains in the wine and may amount to several per cent by weight.

Müller-Thurgau found mannitic bacteria very frequently in the spoiled apple and pear wines of Switzerland.

Müller-Thurgau states that the mannitic bacteria may cause the development of a lactic sour taste in wines, but that other lactic bacteria also usually cause this flavor which is reminiscent of sauer kraut or fermented pickles. Mannitic wines are characterized also by an exceptionally high extract caused by the presence of mannite. In sound, dry wines, the sugar is converted into alcohol and  $\text{CO}_2$  instead of into mannite. Lactic acid, mannite, acetic acid and probably esters formed by the bacteria are all involved in imparting the peculiar "sour-sweet" taste of typical mannitic wines.

In their classical paper (55) on the bacteria of wine, Müller-Thurgau and Osterwalder describe in considerable detail the morphology and cultural characteristics of several strains of *L. mannitopoeus*, namely, *f*, *k*, *p*, *g* and *t*. The first four were isolated from fruit wines, the last from a Gutedel grape wine. In wine fortified with added grape juice, the cultures formed long rods or filaments. On gelatin medium the cells were short. In a yeast extract solution containing levulose, their *L. mannitopoeus f* converted 62–72% of the levulose into mannite. Müller-Thurgau and Osterwalder's organism transformed 13.6% of the levulose into acetic acid and 12.6% into lactic acid. Carbon dioxide was formed and in yeast extract media a mousey odor and taste developed. Lactic and acetic acids, and  $\text{CO}_2$  but no mannite were formed from dextrose and galactose. Some ethyl alcohol was formed from dextrose and galactose, and a mousey taste developed. Sucrose was attacked with formation of lactic acid, acetic acid,  $\text{CO}_2$ , alcohol and mannite; nevertheless, no invert sugar could be found. Maltose was also attacked with formation of lactic and acetic acids, and alcohol. Lactose was not attacked. The organism in this respect differs from the lactic bacteria of milk. Raffinose was attacked. *l*-Arabinose and xylose but not rhamnose were attacked. The mannitic bacillus of Gayon and Dubourg did not attack arabinose. It destroyed malic acid with formation of much lactic and little acetic acid. Tartrates were not affected.

Citric acid but not lactic or succinic was also attacked. These properties are mentioned to indicate the manner in which Müller-Thurgau and Osterwalder differentiated their various organisms.

Five other closely related mannitic bacteria were studied by the same workers. These organisms were characterized by their growth in long, slender filaments, or filamentous chains of slender rods. Like *L. mannitopoeus* these organisms formed mannite, lactic, acetic and  $\text{CO}_2$  from levulose and these acids and  $\text{CO}_2$ , but not mannite from dextrose and galactose. They named the type organism of the group *Lactobacillus gracilis*. Unlike *L. mannitopoeus*, it did not affect cane sugar, maltose and raffinose. Its action on pentose sugars differed from that of *L. mannitopoeus*. These investigators have described two other strains of mannitic bacteria, namely *L. intermedius* from Swiss red wine and *L. gayoni* from an Algerian wine. They resemble *L. mannitopoeus*.

It then appears that the mannitic bacteria of wine can differ among themselves markedly in morphology and in their physiological behavior, but have in common the property of converting levulose into mannite.

As to control and prevention of the mannitic disease in wines, the use of  $\text{SO}_2$  during alcoholic fermentation and subsequent storage of wine will prevent its appearance by inhibiting the bacteria. The organism is sensitive to  $\text{SO}_2$ ; 100 p. p. m. prevents its growth. Pasteurization and germ-proof filtration can also be used.

### 6. The "Bitter Ferment" of Wine

The author has never encountered this disease in the typical form described by Pasteur (63), Voisenet (84) and others. It occurs almost or quite exclusively in red wines and usually develops in old wines several years after bottling, according to European authors. Pasteur observed long rod bacteria, often stained or encrusted with the coloring matter of the wine. The wine becomes turbid and acquires a peculiar odor in addition to the very pronounced bitter taste. Pasteur found no decrease in tartrates, but observed that the glycerol diminished in such wines. Duclaux (28) made a similar observation and concluded that the bitterness is formed from decomposition of the glycerol. Coloring matter precipitates during the course of the disease. Voisenet believed the bitterness due to formation of acrolein resins, the acrolein being derived from the glycerol.

Pasteur heated 100 bottles of Pomard wine of Burgundy of 1863 and 100 bottles were left unheated. All of the former remained clear while all of the latter in time became turbid and finally bitter. The sediment

swarmed with long rod bacteria. This experiment is reasonably good proof that the disease is bacterial.

Mazé and Pacottet (52a) isolated several pure cultures from bitter wines and found them to agree in properties with those of the "mannitic ferment" of Gayon and Dubourg. However, they were unable to reproduce the disease with their pure cultures.

Peroncito and Maggiora (66) claimed to have reproduced the disease by inoculating red wine with pure cultures of bacteria isolated from bitter wines (according to Müller-Thurgau and Osterwalder).

Dahlen (23), and others believed the bitterness might not be due to glycerol decomposition products but to compounds derived from the tannin of the wine. Müller-Thurgau and Osterwalder (55) also incline toward that view and point out that while gallic acid derived from tannin is not bitter, nevertheless the ethyl ether of gallic acid is intensely bitter.

Apparently little recent work has been done on this disease and one is at a loss to decide which, if any, of the various theories as to the origin of the bitter principle, is correct. The disease appears, however, to be caused by rod bacteria similar in appearance to that of the more common lactobacilli of wine.

Voisenet (84) has named the organism isolated by him from bitter wine, *Bacillus amaracrylus*.

### 7. Slimy Wine Spoilage

Occasionally new white wine becomes "ropy" or viscous. Microscopical examination shows the presence of long chains of very short rod bacteria. Pasteur observed these organisms in such wines and reported them as cocci in rosary-like chains.

However, other organisms have been isolated from such wines. Boersch (8) found a sarcina, Aderhold (2) a diplococcus, and Kramer (43) found a bacillus, often in long filaments, that he named *Bacillus viscosus vini*. Mazé and Pacottet (52a) described two rod bacteria from slimy wines that formed filaments and were members of the mannitic bacteria group. Kayser and Manceau (41) have described several short rod bacilli which develop in wine either individually or in chains. Their treatise covers the subject very fully.

The disease is easily prevented by use of 100 p. p. m. of SO<sub>2</sub>, and is cured by addition of tannin and pasteurization. The chemical nature of the slime is not definitely known.

**Bacterium Tartarophthorum.**—Müller-Thurgau and Osterwalder (54) describe a very interesting bacterium from wine that decomposes tar-

trates and tartaric acid and forms in wine  $\text{CO}_2$  and acetic acid. The fixed acid of the wine decreases and the volatile acid increases.

### 8. Other Bacterial Diseases of Wine

White wines often become hazy according to Pacottet, Sannino and others from the presence of *Micrococcus anomalus*. Frequently wines develop a lactic-sour (kraut-like) flavor without becoming mousey in taste ("tourne") and without  $\text{CO}_2$  production (pousse). Lactobacilli of homofermentative species are involved.

### 9. The "Hair Bacillus" of Fortified Wine

Since 1932 the author's laboratory has received many samples of fortified wines of 18–20.5% alcohol content in which an extensive, flocculent, amorphous sediment was evident. At no time does the wine become turbid, unless shaken severely. Chemical examination of such wines reveals ordinarily that very little change in composition has occurred. Upon prolonged storage at room temperature, there is an increase in the volatile and fixed acids with a decrease in sugar content.

Under the microscope the organism responsible for the disease resembles a tangled mass of wet hair in appearance, hence the common name "hair bacillus." It resembles microscopically very strikingly the illustration of Müller-Thurgau and Osterwalder's *Lactobacillus gracilis*. The long filaments are made up of slender rodlike bacteria.

Douglas and Cruess (26) described the disease and the organism briefly, and Douglas and McClung (27) described some of its cultural characteristics. They were unable to obtain growth in any laboratory culture media such as grape juice, tomato juice, meat broth and many others, and resort had to be made to use of agar made with diluted sweet wine in order to obtain colonies for isolation of pure cultures. The organism grows readily and abundantly in diluted sweet wine.

It is sensitive to  $\text{SO}_2$ , 75–100 p. p. m. holding it in check completely. It apparently does not grow in wines of an acidity exceeding 0.50 gm. per 100 cc. as tartaric acid, nor does it grow at pH values below 3.7 nor above 7.0 according to Douglas.

Much bottled fortified wine has in the past developed a cottony deposit of this organism. The wine in such cases was brilliantly clear when it left the winery. Consequently the bottler has been puzzled concerning the origin of what he terms "cottony mold." In such cases the wine may have



been infected in the tank in the cellar, or infected by the bacteria on the inner surfaces of pumps, filling machines, hose lines, tank cars, etc.

The universal means of prevention at present is maintenance of 75 p. p. m. or more  $\text{SO}_2$  in the wine at time of bottling; usually 100 p. p. m. is the concentration desired. This organism spoiled many millions of gallons of wine before the work of this laboratory established effective control measures. It is now very seldom encountered.

### III. Malic Acid Destruction by Bacteria

It has long been known that the acidity of the tart wines of Switzerland and Germany usually decreases quite rapidly and extensively after the alcoholic fermentation, thereby rendering the excessively sour wines of those countries potable. At one time it was thought this decrease was due to precipitation of excess cream of tartar,  $\text{HKC}_4\text{H}_4\text{O}_6$ , but the work of Möslinger (53a), Seifert (74), Koch (42), Kunz (44), Müller-Thurgau and Osterwalder (55) and others showed that the transformation is one involving the conversion of malic acid (a dibasic acid) to lactic acid (a monobasic acid) and  $\text{CO}_2$ , thereby greatly reducing the fixed acidity, and that the organisms usually concerned are micrococci. Seifert described one of these under the name *Micrococcus malolacticus*.

Kunz found as much as 0.4 gm. of lactic acid per 100 cc. in wines in which the malolactic fermentation had occurred; and Möslinger found a maximum of 0.6% lactic in such a wine.

The author has tasted and observed in Swiss cellars new wines undergoing this fermentation. The wines were mildly sparkling from the  $\text{CO}_2$  generated in the process and were of pleasing flavor. Evidently, *M. malolacticus* and *M. acidovorax* (another and similar coccus) impart no or extremely little off flavor to the wine.

In so far as the author is aware, this fermentation is not of great importance in California, but he hastens to say, also, that this condition is, perhaps, more apparent than real, for very little study has been given this problem in California. California wines are low in malic acid as grapes ripen completely here; whereas in Switzerland they do not ripen thoroughly and therefore contain much free malic acid. Green grapes of any region are rich in malic which decreases and may almost completely disappear during ripening.

Müller-Thurgau and Osterwalder (55) studied this fermentation and the organisms responsible for it very thoroughly. They mention that Pasteur considered a coccus-like bacterium as the cause of mellowing of wines.

Their micrococci were classified as members of two species, namely, *M. acidovorax* and *M. variococcus*. The former was isolated from a pear wine and several strains of the latter from grape wines from several districts in Switzerland.

*M. acidovorax* grown in white wine was grouped in tetrads frequently, less frequently in triads and as diplococci. Its usual size was 0.5 to 0.7  $\mu$ . *M. variococcus* was larger, 0.75 to 1.0  $\mu$  in diameter and often occurred in larger groups than tetrads, also as tetrads, diplococci and individual cells.

The two species showed distinct morphological differences in other media.

*M. acidovorax* decomposes lactose energetically and maltose quite rapidly, whereas *M. variococcus* does not attack lactose at all, and maltose only slightly. *M. variococcus* attacks amygdalin while *M. acidovorax* does not.

Seifert's *M. malolacticus* agrees with Müller-Thurgau and Osterwalder's *M. variococcus* in size but differs markedly from it in its action on levulose and dextrose.

The three organisms resemble each other closely in their action on malic acid, citric, tartaric, lactic and succinic acids. They attack malic acid, and malates vigorously, but not the other acids or their salts.

They differ from most other wine bacteria such as *L. manniopoeus*, *L. gracilis*, *L. hilgardii*, etc., in producing very little acetic acid in wines, and as they do not adversely affect the flavor and quality of the wine, they are considered benefactors to the wine industry of Switzerland and Germany.

#### IV. Role of Enzymes in Wine Making

The importance of certain enzymes in wine making is recognized by most authors on enology and wine production. Amerine and Joslyn (1) call attention to the fact that alcoholic fermentation itself in the final analysis is an enzymic process. They also call attention to the roles of pectic enzymes and oxidases.

##### 1. Oxidase of the Grape

Martinand (50) appears to be the first to report that darkening of wines is caused by oxidation, catalyzed by a soluble ferment termed "diastase" which in a later paper (51) he considered to have the properties of a lac-case. Since mold growth is frequent on French grapes, it is possible that he may have confused the oxidizing enzyme of *Botrytus cinerea* with that of the grape tissues.

Pacottet (62) states that grapes and wines contain an oxidase naturally

occurring in grape tissues, but that this oxidase is much less abundant and of less importance than that from the gray mold, *Botrytus cinerea*, of French grapes. Ventre (83) makes similar statements.

Recently Hussein and Cruess (38) have extended previous knowledge of the oxidase of grapes. They found that the oxidase, prepared by precipitation with acetone in citrate buffer of pH 4.5, reprecipitation with alcohol and solution in citrate buffer solution, would oxidize the purified natural substrate from grapes causing a browning of the color. Since  $H_2O_2$  was not necessary for this oxidation, the natural substrate apparently contained an orthodihydroxy linkage, such as exists in catechol, caffeic acid and certain tannins. The orthodihydroxy compounds act as organic peroxides of Onslow (59). The enzyme also oxidized many phenolic oxidase indicators in the presence of  $H_2O_2$  or of added catechol. Its optimum pH was 4.5–5.5. It was very sensitive to KCN, *e. g.*,  $5 \times 10^{-6}$  KCN destroyed over 90% of its activity and  $4 \times 10^{-4}$  destroyed it completely, indicating it to be a true peroxidase. There was no evidence that a dehydrogenase existed in the grapes used. It destroyed ascorbic acid in the presence of catechol, no darkening of the latter occurring until all the ascorbic acid had been destroyed. It was completely inhibited by 60% of alcohol by volume but retained practically full activity at 10 to 20%, the normal range of wines. It was sensitive to  $SO_2$ . Papain and trypsin destroyed the enzyme, indicating a protein character.

In another paper Hussein and Cruess (38) report that the naturally occurring grape oxidase hastens the browning of the color of white dry wine in the presence of oxygen, also that increasing the acidity of the wine greatly diminished the rate of enzymic browning. However, their wines also browned considerably by auto-oxidation after destruction of the enzymes by heat.

## 2. *Botrytus* Mold Oxidase and Oxidasic Casse

In most European wine-producing regions grapes often develop a gray mold, *Botrytus cinerea*, that penetrates the skin and flesh of the berries and brings about a desirable concentration of the sugar in the must by evaporation of water from the mold's mycelium. At the same time, the mold secretes a powerful oxidase, responsible for excessive browning and clouding of white wines and precipitation of the color of red wines.

Laborde (46) showed the relation between the presence of *Botrytus cinerea* on grapes and the oxidase content of the wine. Pacottet (62) describes methods of controlling and of destroying this oxidase in wine. The

addition of  $\text{SO}_2$  or bisulfites will hold it in check, a procedure in very common use in France and Germany. It may be destroyed by heating the wine to  $80^\circ \text{C}$ . for about 3 min., according to Ventre (83). The writer regrets that he has had no experience with this enzyme, as *Botrytus cinerea* does

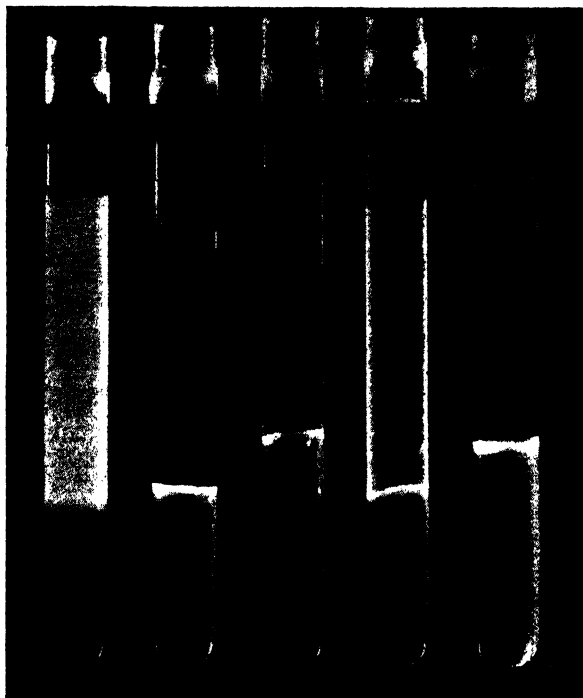


Fig. 13.—Effect of several pectic enzymes on grape must. No. 1: untreated; Nos. 2, 3 and 5 treated with commercial enzymes; No. 4: untreated.

not usually grow on California grapes. It requires wet, rainy weather during the picking season, whereas the California weather at that time is usually very dry and warm.

### 3. Pectic Enzymes

The natural clearing of wines after fermentation is probably in part due to the clotting action of enzymes on wine colloids, particularly of pectic enzymes on the natural pectic substances of wine. Besone and Cruess

(4) report experiments tending to substantiate this belief; unheated juice and wine made from it cleared perfectly, that which was heated to destroy enzymes remained cloudy. Pectic enzymes added to the latter caused rapid clearing.

They found commercial pectic enzymes very useful in the clarification of new wines. Evidently, pectin is hydrolyzed to pectic acid which then separates as a clot or precipitate easily removed by settling or filtration. At the same time, it evidently carries with it finely divided, suspended particles in the wine. It was not inhibited by the  $\text{SO}_2$  normally used in wine making nor by the 20% of alcohol found in fortified wines. In a small industrial scale test the enzyme-treated new wine cleared in two days; the untreated was still hazy after four months' storage.

It was found that the enzyme can be added to advantage before fermentation and that it then accomplishes its effects during fermentation, bringing about rapid clearing after fermentation is complete.

#### 4. Other Enzymes

Amerine and Joslyn state that wines probably contain tannase. Since *Vitis vinifera* varieties of grapes contain only invert sugar, whereas *Vitis labrusca* varieties contain both sucrose and invert sugars, it is possible that *V. vinifera* grapes all contain invertase. Yeasts, of course, contain invertase in addition to the enzymes involved in alcoholic fermentation. It is possible that through autolysis of the yeast following cessation of fermentation small amounts of some enzymes of the yeast are liberated into the wine. The writer has been unable to find any references on that point, however.

Whether or not grapes contain ascorbase, appears not to have been definitely settled. The work of Morgan, Nobles, Wiens, Marsh and Winkler (52b) shows that the vitamin C content of the fresh grapes is lost in wine making and aging. This loss may or may not be due to simple auto-oxidation. Grapes are low in vitamin C.

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